From the DEPARTMENT OF LABORATORY MEDICINE

Karolinska Institutet, Stockholm, Sweden

MHC CLASS I RESTRICTED AND ANTIGEN-SPECIFIC TCRS AGAINST VIRAL AND TUMOR-ASSOCIATED ANTIGENS (TAAS) FOR FUTURE BIOLOGICAL THERAPY

Zhenjiang Liu

刘振江

Karolinska Institutet

Stockholm 2017

MHC class I restricted and antigen-specific TCRs against viral and tumor-associated antigens (TAAs) for future biological therapy

THESIS FOR DOCTORAL DEGREE (Ph.D.)

Inghesalen, Tomtebodavägen 18 A, Solna Campus, Karolinska Institutet,

Monday May 8th , 2017 at 13:30

By

Zhenjiang Liu

Principal Supervisor:
Professor Markus Maeurer, MD, PhD
Karolinska Institutet
Department of Laboratory Medicine
Division of Therapeutic Immunology

Co-supervisor(s):
Prof. Olle Ringden MD, PhD
Karolinska Institutet
Department of Laboratory Medicine
Division of Therapeutic Immunology

Professor Jonas Mattsson MD, PhD
Karolinska Institutet
Department of Oncology-Pathology

Associate Professor Michael Uhlin MD, PhD
Karolinska Institutet
Department of Oncology-Pathology

Opponent:
Professor Horacio L. Rilo, MD, PhD
Hofstra Northwell School of Medicine
Pancreas Disease Center

Examination Board:
Professor Ola Winqvist MD, PhD
Karolinska Institutet
Department of Medicine
Division of Translational Immunology Unit

Professor Per Øyvind Enger MD, PhD
University of Bergen
Department of Biomedicine

Associated Professor Per Almqvist MD, PhD
Karolinska Institutet
Department of Clinical Neuroscience (CNS)

献给我的家人

Two things fill me with constantly increasing admiration and awe, the longer and more earnestly I reflect on them: the starry heavens without and the moral law within.

Immanuel Kant

笛里关山，樽前日月，回首空凝伫。吾今未老，何须清泪如雨。

高启

ABSTRACT

Adoptive cellular immunotherapy (ACT) refers to the process of transferring immune cells (autologous or allogeneic) directly to the host as a treatment for cancer or infectious diseases. The effector cells could be antigen-specific, like T cells, or non-specific, such as NK cells or lymphokine-activated killer (LAK) cells. A substantial amount of evidence shows that T cells play an important role in controlling pathogens and tumor growth. Controlling pathogens and orchestrating the function of immune cells depends on engagement of the nominal TCR (T-cell receptor) with its ligand (the MHC class I or -II peptide complex), the structure and function of T cell receptor (TCR) and the subsequent immune effector functions upon TCR triggering (cytotoxicity, proliferation, cytokine production).

Tumor-infiltrating T-cells (TILs) represent a source of T-cells for the immunotherapy of patients with tumors of the central nervous system and pancreas. According to the results of **paper I and II**, we successfully established a rapid TIL expansion protocol for patients with brain tumors or pancreatic cancer. TILs were shown to produce Th1-cytokines and were able to recognize autologous tumor cells defined by cytokine production or cytoxicity.

In **paper III**, we found that tumor associated antigens (TAAs)-reactive T-cells could be successfully expanded from patients with glioma with IL-2, IL-15 and IL-21; they exhibit a Th1 cytokine pattern and a central memory phenotype. NY-ESO-1 expression was found in 15/38 cases and survivin expression in 20/40 cases in glioblastoma, defined by immunohisto-chemistry. Thus, NY-ESO-1 or survivin represent a potential target for anti-NY-ESO-1 or anti-survivin directed T-cells for the biological therapy of patients with glioblastoma (GBM).

Mesothelin was first identified to be overexpressed in ovarian cancer. It is constitutively expressed in normal tissue, e.g. pericardium, pleura or peritoneum. This 40kDa protein could serve as a tumor marker and as a target of immunotherapy for anti-cancer directed T-cells. In **paper IV,** mesothelin was found to be expressed in 4 out of 11 GBM tissues, by immune-fluorescence staining. Mesothelin directed T cell reactions were also observed in a whole blood assay (WBA) measured in 293 patients with brain tumors. Mesothelin immunogenic epitopes were also identified using a peptide mapping assay; mesothelin-specific TILs could be expanded from glioma samples.

We analyzed in detail potential prospective factors in patients with GBM (n=145) and non-GBM (n=60) which refers to glioma (WHO grade II or III). In **paper V**, we performed univariate Kaplan-Meier (K-M) survival analysis by setting of groups based on demographic, clinical, immunological parameters and immunological reactivity patterns, then we defined factor(s) with a cut-off strategy. We performed further multivariate analysis with a Cox proportional hazards model (forward and backward stepwise analysis) to determine the key factor related with patient's survival by considering (and omitting) interactions between employed factors. We found that T-cell reactivity to an individual survivin epitope (97-111) is positively related (P=0.024) with survival of patients with GBM. The same was found to be true for the serum cytokine pattern of IL-4/IL-5/IL-6 (P=0.052) and IFN-γ/TNF-α/IL-17A (P=0.003) which could serve as 'predictor' for prognosis in clinical settings. The cytokine serum profile as well as the immune reactivity to survivin may serve as a clinically relevant indicator for the clinical follow up of patients with GBM after surgery and provide a viable option to offer tailored immunological therapy.

LIST OF SCIENTIFIC PAPERS

I. **Zhenjiang Liu**, Qingda Meng, Jiri Bartek Jr, Thomas Poiret, Oscar Persson, Lalit Rane, Elena Rangelova, Christopher Illies, Inti Harvey Peredo, Xiaohua Luo, Martin Vijayakumar Rao, Rebecca Axelsson Robertson, Ernest Dodoo, Markus Maeurer. **Tumor-infiltrating lymphocytes (TILs) from patients with glioma.** Oncoimmunology, Volume 6, 2017-Issue 2.

II. Qingda Meng, **Zhenjiang Liu**, Elena Rangelova, Thomas Poiret, Aditya Ambati, Lalit Rane, Shanshan Xie, Caroline Verbeke, Ernest Dodoo, Marco Del Chiaro, Matthias Lohr, Ralf Segersvärd, Markus J. Maeurer. **Expansion of tumor-reactive T cells from patients with pancreatic cancer.** J Immunother Volume 39, Number 2, February/March 2016.

III. **Liu Zhenjiang**, Thomas Poiret, Oscar Person, Qingda Meng, Lalit Rane, Jiri Bartek Jr, Julia Karbach, Hans-Michael Altmannsberger, Christopher Illies, Xiaohua Luo, Inti Harvey Peredo, Elke Jäger, Ernest Dodoo, Markus Maeurer. **NY-ESO-1 and Survivin expression in tumor tissue. Antigen-specific T-cell responses in PBMCs from patients with glioma**. Submitted to Cancer Immunology. Immunotherapy, in revision.

IV. **Liu Zhenjiang**, Martin Rao, Thomas Poiret, Silvia Nava, Oscar Persson, Qingda Meng, Anna von Landenberg, Jiri Bartek Jr, Shanshan Xie, Inti Harvey Peredo, Ernest Dodoo, Markus Maeurer. **Mesothelin as a novel biomarker and immunotherapeutic target in human glioblastoma multiforme**. Oncotarget, in revision.

V. **Liu Zhenjiang**, Martin Rao, Elisabeth Sandberg, Xiaohua Luo, Jiri Bartek Jr, Davide Valentini, Esther Schoutrop, Anna von Landenberg, Qingda Meng, Thomas Poiret, Oscar Persson, Nina Hoffmann, Lalit Rane, Julia Karbach, Hans-Michael Altmannsberger, Christopher Illies, Elke Jäger, Inti Harvey Peredo, Ernest Dodoo, Markus Maeurer. **Survivin peptide-specific cellular immune responses and cytokine networks predict the improved survival of patients with glioblastoma multiforme.** Manuscript.

LIST OF ABBREVIATIONS

ACT	Adoptive cellular immuno-therapy
APC	Antigen-presenting cell
CAF	Cancer-associated fibroblastic cell
CTA	Cancer-testis antigen
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated antigen-4
DC	Dendritic cell
EGFRvIII	Epidermal growth factor receptor variant 3
ELISA	Enzyme-linked immunosorbent assay
GBM	Glioblastoma multiforme or glioblastoma
GPI	Glycophosphatidylinositol
HLA	Human leukocyte antigen
ICS	Intracellular cytokine stain
IDH	Isocitrate dehydrogenase
IFN-γ	Interferon-γ
IHC	Immunohistochemistry
IHC	Immunohistochemistry
IL-10	Interleukin-10
IL-15	Interleukin-15
IL-17A	Interleukin-17A
IL-2	Interleukin-2
IL-21	Interleukin-21
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
MDSC	Myeloid-derived suppressor cell

MHC	Major histocompatibility complex
MPF	Megakaryocyte-potentiating factor
NK cell	Nature killer cell
OS	Overall survival
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PDA	Pancreatic ductal carcinoma
TAA	Tumor associated antigen
TCM	Central memory T cell
TCR	T cell receptor
TEM	Effector memory T cell
TEMRA	Terminally differentiated effector memory T cell
TGF-β	Transforming growth factor beta
TILs	Tumor infiltrating lymphocytes
TNF-α	Tumor necrosis factor-α
Treg	Regulatory T cell
WBA	Whole blood assay

1 INTRODUCTION

'Self-recognition and non-self-elimination' is the function of immune system which refers to the multi-faced interplay of different parts of the immune system on the levels of organs, cells and molecules. Throughout the body, two groups of immune organs are defined: 'primary' or 'central'; the first including the thymus and bone marrow, where immune cells have been generated. The latter refers to the 'secondary' or 'peripheral' immune system, which encompasses: lymph nodes, appendix, Peyer's patches, tonsils, adenoids, spleen and Mucosal-associated lymphoid tissue (MALT). Immune molecules, such as cytokines or defensins are mainly produced by immune cells and act as nodes in the formation of immune networks. From the aspect of evolution and specificity, the immune system could be classified into two systems: the innate immunity, also called natural immunity, refers to the function of cells or proteins which are consistently present and allow an immune response to pathogens without a refractory period; the reactions of innate immunity peak within 12 hours after engagement; long term protection is not provided. Epithelial barriers, phagocytes, dendritic cells, NK cells and complement are important constituent parts of this system [1]. After 12 hours, adaptive immune response or 'acquired immunity' will continue to eliminate pathogens which could overcome innate immune defenses in the first place. B- or T- lymphocytes are responsible for the antigen specific reactions during period of adaptive immunity, they constitute humoral immunity and cell-based immunity, respectively. Immunological memory is also formed in this duration to form long term protection [2].

The immune system may be categorized into three areas: i). immunosurveillance, ii). immuno-homeostasis, iii). immuno-defense. Immuno-defense refers to the recognition of microbes as 'foreign' followed by successful elimination of the foreign microbes. The capability of immune system in maintaining stability of the inner micro-environment is called immuno-homeostasis [1]: the immune system could recognize and remove dead or damaged cells. Recognition and elimination of transformed cells by immune system is called immune-surveillance [2]. Tumor immunogenicity establishment by the host immune system is actually a balance between cancer immune responses and tumorogenesis [3], the active interplay of transformed cells and host immune cells.

Glioma arises from glia cells in the central nervous system (CNS) [4]. There are four glioma grades according to the WHO classification system, which can be classified as low (Grade I

and II) and high (Grade III and IV) grade groups. Within the low grade group, grade I glioma is histologically benign with a low potential for malignant progression, while grade II is associated with an increased risk to high grade progression. Grade III and IV gliomas are defined as malignant brain tumors. Grade IV glioma represents glioblastoma multiforme (GBM), an aggressive brain tumor with poor prognosis and a medium survival of 14.6 months, a survival value which did not change significantly during the last 20 years [5].

Pancreatic cancer, especially pancreatic ductal adenocarcinoma, is often associated with late diagnosis, frequent metastasis, resistance to chemotherapy and radiation therapy, and a poor prognosis which did not significantly change for the last decades. The 5-year survival rate of PDA is as low as 1%–4% and the median survival of patients with unresectable tumor is around 4-6 months [6].

This thesis will focus on tumor specific T cells which could be expanded from tumor (glioma or pancreatic cancer) tissue or from the patients' peripheral blood mononuclear cells (PBMCs) for potential clinical immunotherapy. Tumor infiltrating lymphocytes (TILs) are derived from fresh brain tumor tissue and could be expanded to meet the requirement for clinical therapy. TILs are often tumor specific and functional in elimination of tumor cells; a perfect candidate for adoptive cellular immunotherapy (ACT) targeting tumor mutations.

In this thesis, we define NY-ESO-1，Survivin and Mesothelin as TAAs and also as potential targets for cellular therapy. NY-ESO-1 was identified in 1997 by Chen et al with a SEREX technique, screening a tumor cDNA library with sera from cancer patients [7]. NY-ESO-1, 'NY' refers to New York, 'ESO' stands for esophageal carcinoma, '1' means firstly identified as a new gene family, also named as cancer/testis antigen 1B (CTAG1B) or the L antigen family member 2 (LAGE-2), which is broadly expressed in many kinds of cancers (around 30-40% tumors) and shows a high degree of immunogenicity. Survivin, (baculoviral inhibitor of apoptosis repeat-containing 5, BIRC5) has a central role in apoptosis, cell cycle, e.g. and according to its name, it plays a critical role in tumor cell apoptosis inhibition and tumor survival. Survivin could serve as a potential target for cancer therapy, some of the peptides derived from survivin are highly immunogenic [8, 9]. Mesothelin, encoded by the MSLN gene, is overexpressed mainly in pancreatic adenocarcinoma (~100%), ovarian cancer (70%), lung adenocarcinoma (50%) or mesothelioma (~100%) as a tumor differentiation antigen [10]. It is derived from a 71kDa protein which anchors to the cell surface with glycophosphatidylinositol (GPI) and contains a shed protein (31kDa) called megakaryocyte

potentiating factor (MPF). In certain conditions, the mesothelin protein is shed and detectable in serum. Mesothelin can be recognized as a target for immunotherapy due to its limited expression in normal tissue and high expression in tumor lesions.

1.1 TUMOR BIOLOGY OF GLIOMA

1.1.1 Tumor classification and genetic/molecular features

Among all primary brain tumors, glioma account for around 26% of tumors located in the central nervous system. [11]. Based on the 2007 World Health Organization (WHO) classification system (2007 CNS WHO), pathological characteristics, which are commonly used in brain tumor grading system include: atypical cells, proliferation, mitosis and necrosis. Gliomas without any of these pathological changes are classified as 'grade I' which mainly occurs in the pediatric population with a low potential of malignant progression. Certain brain tumors like ganglioglioma, dysembryoplastic neuro-epithelial tumor (DNET), pilocytic astrocytoma (PA) and pleomorphic xanthoastrocytoma (PXA) belong to this group [12]. With only one of the characteristics, grade II gliomas could be histologically divided into 3 subtypes due to their origin: astrocytoma, oligodendroglioma and oligoastrocytoma. The prognosis of grade II astrocytoma is significantly poorer than oligodendroglioma with a median overall survival (5.6 years versus 11.6 years) and an increased risk of progression to high grade glioma progression (75% versus 45%) [13]. A frequent driver mutation within all 3 subtypes is isocitrate dehydrogenase (IDH) R132 which can induce the generation of 2-hydroxyglutarate (2-HG) that plays a role in tumorigenesis [14]. Beside the IDH mutation, co-mutations in different histological subtypes such as 1p/19q deletion, the capicua transcriptional repressor (CIC) mutation and FUSE binding protein1 (FUBP1) mutations in oligodendroglioma, or the TP53 loss/mutation and X-linked alpha thalassemia /mental retardation syndrome (ATRX) in astrocytoma effect the clinical outcome [15].

Anaplastic (WHO grade III) gliomas with 3 subtypes (anaplastic astrocytoma/oligodendro-glioma /oligoastrocytoma) could be either primary, without any history of low grade gliomas or secondary to progression of low grade gliomas. Similar to grade II gliomas, the difference in histology is associated with a different 5 year survival (26% in anaplastic astrocytoma versus 50% in anaplastic oligodendroglioma) and an increased risk to GBM progression. Besides the mutation profile histology, grade III gliomas contain more specific co-mutations. For instance, the loss of phosphatase and tensin homolog (PTEN), retinoblastoma 1 (RB1) loss, cyclin-dependent kinase (CDKN2A) loss and cyclin dependent 4 or 6 (CDK4/6) amplification could be found in anaplastic astrocytoma (1.7% of primary brain tumor) while phosphatase and tensin homolog (PTEN) loss, cyclin-dependent kinase (CDKN2A) loss and

telomerase reverse transcriptase (TERT) promotor mutation could be found in anaplastic oligodendroglioma (0.6% of primary brain tumors) [15-17].
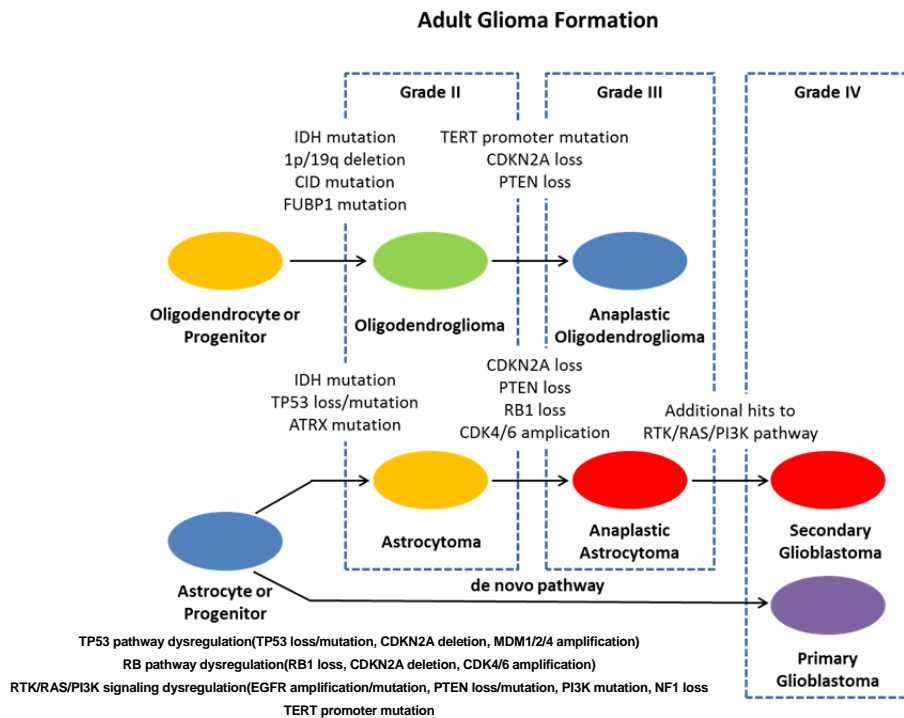
Adult Glioma Formation

Figure 1. Driver events in adult gliomagenesis. RTK, receptor tyrosine kinase; PI3K, phosphoinositide 3-kinase; EGFR, epidermal growth factor receptor; NF1, neurofibromin 1. *Source: DeVita, Hellman, and Rosenberg's Cancer: Principles & Practice of Oncology, 10edition. (2015). Section 10. Neoplasms of the Central Nervous System:96. Molecular Biology of Central Nervous System Tumors:1403-1410.*

Different from the 2007 CNS WHO, an updated version (rather than a formal new edition), has been summarized in May 2016 with major changes concerning tumor classification: Molecular genetic features and histology parameters have been combined to generate a higher diagnostic accuracy. Beside the traditional light microscope features, newly genotypic parameters include IDH wild-type/mutation, 1p/19q co-deletion, TP53 mutation and ATRX loss, which serves as a key or 'driver mutation' in diffuse or anaplastic astrocytoma or oligodendrglioma, and even GBM. It should be noted that the diagnosis of oligoastrocytoma or anaplastic oligoastrocytoma according to the 2007 CNS WHO, which was difficult to define objectively, and caused high interobserver discordance, does no longer exist. With the new criteria, most of the oligoastrocytoma cases could be classified as either astrocytoma or oligodendroglioma, the rare and true oligoastrocytoma cases are categorized in the 'not otherwise specified (NOS)' group. A similar strategy could be followed for anaplastic oligoastrocytoma [18].

As the most common malignant brain tumor, glioblastoma (WHO grade IV), which accounts

for around 16% of all primary brain tumors and around 60% of gliomas [19], can be classified into 3 groups according to 2007 CNS WHO: IDH-wildtype glioblastoma, IDH-mutant glioblastoma and glioblastoma NOS. IDH-wildtype glioblastoma is the synonym of primary glioblastoma which refers to the tumorigenesis from a de novo pathway, without evidence of a precursor lesion. Tumors which occur through progression of low grade gliomas are named as 'secondary' or 'IDH-mutant glioblastoma'. Differences in key characteristics such as age, tumor incidence, mutation profiles and patient survival are shown in **Table 1** [18].

The adult glioma formation from low grade to high grade is provided in **Figure 1** according to the database of The Cancer Genome Atlas (TCGA) project.

1.1.2   Hallmarks of glioma

Hallmarks of cancer were well-defined by Hananhan and Weinberg in 2011 in a Cell review which provided the framework for understanding the biological capabilities of neoplastic diseases and breakthrough points for cancer treatment [20]. Genomic instability and mutation as a hallmark is now a well accepted concept in oncology and /or in onco-immunology.

	IDH-wildtype GBM	IDH-mutant GBM
Synonym	Primary GBM, IDH-wildtype	Secondary GBM, IDH-mutant
Precursor lesion	Not identifiable; develops de novo	Diffuse astrocytoma Anaplastic astrocytoma
Proportion of GBMs	~90%	~10%
Median age at diagnosis	~62 years	~44 years
Male-to-female ratio	1.42:1	1.05:1
Mean length of clinical history	4 months	15 months
Median overall survival		
-Surgery+radiotherapy	9.9 months	24 months
-Surgery+radiotherapy+chemotherapy	15 months	31 months
Location	Supratentorial	Preferentially frontal
Necrosis	Extensive	Limited
TERT promoter mutations	72%	26%
TP53 mutations	27%	81%
ATRX mutations	Exceptional	71%
EGFR amplification	35%	Exceptional
PTEN mutations	24%	Exceptional

Table 1. Characteristics of IDH-wildtype/mutant glioblastomas. *Source: The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. Acta Neuropathol (2016) 131:803–820.*

1.1.2.1 Cancer stem cells (CSCs)

Cancer stem cells (CSCs) refer to the ability of self-renewal, they act similar as compared to hemotopoietic stem cells, yet they exhibit malignant biological behavior. The role of CSCs is increasingly accepted in gliomas, especially in GBM initiation, progression, angiogenesis; it has also biological meaning for treatment (i.e. radiotherapy or chemotherapy) resistance [21]. Although the existence of CSCs is well accepted, the challenge remains how to distinguish CSCs from 'normal CNS stem cells' or common cancer cells, defined by morphological aspects or by genetic/molecular fingerprints [22]. Some shared markers like CD133 (Prominin-1), oligodendrocyte lineage transcription factor 2 (OLIG2), inhibitor of differentiation protein (ID1) or Nestin could be aid to identify CSCs. A list of well accepted optional markers, which could be used in CSCs identification, either by flow cytometry or by detection on the gene/protein level includes: CD133, CD44+ID1, stage-specific embryonic antigen-1(SSEA-1)/Lewis X/CD15, neuronal cell adhesion molecule L1CAM/CD171, Integrin α6 or A2B5(ganglioside marker) [22, 23]. CD133, the first and most predominant marker for glioma CSCs, is a penta-span transmembrane glycoprotein encoded by the PROM1 gene, with its function in proliferation and differentiation. Since the mRNA level of CD133 is not directly related with 'stemness', so other components served as marker, e.g. the glycosylated form of CD133 [24]; however, some CSCs could still be CD133 negative [25]. Equipped with a sensor to monitor subtle changes within the CNS environment, CSCs are able to self-regulate via intrinsic and extrinsic mechanisms [22]. Intrinsic factors refer to genetic and epigenetic mechanism, metabolism regulation; while extrinsic factors refer to niche factors (signal pathways activated by environment molecules and receptors), immune modulation and the formation of 'tumor microenvironment'.

1.1.2.2 Tumor Heterogeneity

Similar to human society and other organism communities, the 'diversity' of individuals within the population could contribute to the stability, facing a changeable and diverse environment. This kind of diversity is of significance for the whole population. Similarly, the diversity of tumor cells is reflected in their profile and biological behavior, such as morphology, proliferation ratio, invasion ability, metastatic capabilities and treatment resistance. The intuitive clue of GBM morphological features could be observed through the light microscope: atypical cellular structures with nuclear polymorphisms, co-existence of heterogeneous cell populations. The pathologist use the word 'multiforme' to describe the

high cellular nonuniformity [26]. There are a plenty of examples for molecular heterogeneity in glioma: a). Remarkable differences among cells from the same tumor lesion or established tumor cell line [27]. b). Chromosomal aberration disparity among different areas of the same tumor [28]. c). Expression of EGFRvIII is limited to certain subgroups of tumor cells, rare cases with broad EGFRvIII expression [29]. d). Uneven distribution and expression of O6-methylguanine–DNA methyltransferase (MGMT) within one GBM tumor lesion [30]. The origin of heterogeneity in glioma, can be exploited by alternative models: (1). Clonal evolution: even though with monoclonal initiation, mutations accumulated during tumor progression would induce the state of genetic chaos, 'tough' clones who could survive under selective pressure such as hypoxia, chemo-therapy or radiotherapy will give rise to a heterogeneous population [31]. (2). CSCs model: under selective pressure as a driving force, CSCs would be altered to adapt to the changeable intrinsic or extrinsic environment. At the same time; CSCs never stop to give rise to progeny during the progression, a series of daughter cells with distinctive biological fingerprint will be the basis of heterogeneity [32]. (3). Cell plasticity: epigenetic regulation could induce tumor cells with the same initiation into different behavior and capacity, like drug resistance. (4). Cell-to-cell interaction: cross talk between tumor cells via physical contact or network established functional molecules could enhance the survival of the whole tumor population, which would cause retained tumor diversity [33].

1.1.2.3 Angiogenesis, invasion and metastasis

An 'angiogenetic switch' is activated and remains 'on' during the process of glioma-genesis in order to enhance delivery of nutrients or tumor cell migration. The glioma vascular structure is remarkably disorganized due to angiogenesis, which would cause further hypoxia, heterogeneity and drug resistance. The formation of a vascular niche with CSCs could give rise to other tumor lesion [34]. However, this could also be seen as a therapeutic target. 'The angiogenetic switch' is controlled by key regulators such as signaling proteins, oncogene expression or inflammatory cytokines derived from immune cells (**Figure 2**). Vascular endothelial growth factor (VEGF) has been well described and is a well characterized signaling protein and angiogenesis inducer, whose expression is tightly related with hypoxia and acidosis. By binding with its receptors (VEGFR-1-3), VEGF could orchestrate the epithelial proliferation and migration via the hypoxia inducible factor 1(HIF-1)/VEGF-A pathway, and induce increased permeability of vasculature structure which could cause

elevated interstitial pressure and edema [35]. Additionally, interleukin-8 (IL-8) from microglia cells could partly be responsible for hypoxia condition [36].

Unlike other kind of malignant solid tumors, glioma metastasizes through extracellular routes, mainly instead of the intravascular or lymphatic system. Tumor cells could also migrate, guided by vasculature or nerve bundles which is termed 'perineural invasion'. Glioma tumor cells migrate to form local or distant satellite lesions, or even mirror lesion in contralateral hemisphere, but metastasis outside the brain rarely exists [37]. Briefly, 4 steps are involved in tumor invasion [35]: a). Invading cells detachment. Down regulation or inactivation of cadherin, functional in cell-to-cell junction formation and stable structure maintenance, is thought to play a key role in this stage. b). Extracellular matrix (ECM) adherence. Integrin expression on glioma cells could induce adherence to ECM and upregulation of integrin on glioma cells. c). ECM degradation. Proteases, such as matrix-metalloproteinase (MMP), are produced by glioma cells and play a part in ECM degradation. This process is tightly related with the transcription factor NF-κB [35]. d). Motility and contractility. Similar to the migration pattern of stem cells in the mature brain or non-transformed neural progenitors during embryonic development cytoplasmic mediators, like myosin induce contractility capable of altering the shape of cells.

Figure 2. Angiogenetic switch. *Source: The scientific contributions of M. Judah Folkman to cancer research. Nature Reviews Cancer 8, 647-654.*

1.1.2.4 Apoptosis resistance and survival signaling

Apoptosis resistance and survival signaling in glioma could represent two sides of one coin. It refers to distinct processes in glioma-genesis. The apoptosis signaling network includes an extrinsic and intrinsic pathway [38]: as a death signal comes from outside the cell, like TNF-α, FasL or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) binding to their respective receptors TNFR1, CD95 or death receptor (DR)4/5, the intracellular death domain recruitment, continues with activation of the caspase cascade leading to DNA fragmentation and chromatin condensation. The intrinsic pathway is triggered by intracellular signals like
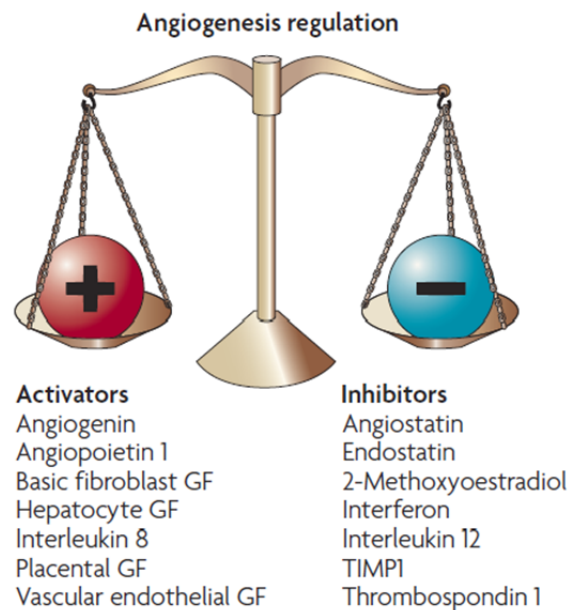
oxidative stress, DNA damage or insufficient growth factors. Proteins of the B cell lymphoma family are involved in the intrinsic pathway regulation [39]. Dysregulation of intrinsic or extrinsic pathway causes apoptosis resistance. For instance, BCL-2 and BCL-X_L, which belong to anti-apoptotic BCL-2 family, could bind with pro apoptotic BCL-2 proteins and therefore inhibit the programmed death. Up-regulation of the anti-apoptotic BCL protein or down-regulation of pro-apoptotic protein would induce tumor growth. Then survival signaling pathways in glioma are cross-linked as a complex network. For instance, EGFR or platelet-derived growth factor receptor (PDGFR) binding induces receptor tyrosine kinase (RTK)/RAS/ Phosphtaidylionositol-3-kinase (PI3K) signaling dysregulation, the RB dysregulated pathway as well as the dysregulated TP53 pathway [40, 41].

1.2 TUMOR BIOLOGY OF PANCREATIC CANCER

1.2.1 Epidemiology, etiology and classification

Pancreatic cancer arises from cells either from the exocrine (around 99%) or endocrine component within the pancreas. Pancreatic ductal adenocarcinoma (PDA) or pancreatic adenocarcinoma (PA), accounts for about 85% of pancreatic cancer. Pancreatic cancer is one of the most lethal tumors: it is reported that within 44,000 individuals with newly diagnosed pancreatic cancer in the US, around 80% of patients will succumb to the disease within one year [42]. Over the past 80 years, the death rates of most cancers exhibited a remarkable decrease, while the 5 year survival rate of PDA is still less than 5%, similar to that in the 1980s and the 2000s. Even for patients (15-20%) who undergo curative resection, only 20% of them survive after 5 years, with no significant change in disease specific survival in the past 40 years [43]. Briefly, PDA is high aggressive with profound treatment resistance and poor prognosis. It takes many years for tumor progression, so with a median age at 71, PDA often occurs in the elderly population and 74% of patients are within the range of 55 to 84 [44].

According to the American Joint Committee on Cancer (AJCC), 7th edition, pancreatic cancer is staged from 0 to IV based on the TNM system. Almost all of the pancreatic cancers are from epithelial cells, lymphoid neoplasms or primary mesenchymal tumors (e.g., sarcomas) are rare. 98% of pancreatic could be categorized according the gross appearance: solid neoplasm which includes: PDA/PA, pancreatic endocrine neoplasm, pancreatoblastoma and acinar cell carcinoma; cystic neoplasm which includes intraductal papillary mucinous neoplasms (IPMN), mucinous cystic neoplasms (MCN) and solid-pseudopapillary neoplasm [45].

Risk factors of pancreatic cancer could be grouped in three categories: genetic, environmental and medical risk factors. A positive family history, especially with a first degree case (parents, offspring or siblings) is a clear-cut risk factor; around 5-10% of pancreatic cancer is familial, yet genetic defects which had been discovered could only explain10-15% familial cases [46]. Concerning environmental factors, tobacco represents an independent risk factor of PDA, it takes as long as 20 years before the risk returns to baseline after smoking cessation [47]. Interestingly, cigars are also considered as a risk factor, while smokeless tobacco is not, the case is not clear for environmental smoke, such as air pollution or passive smoking.

Occupational hazards, like chlorinated hydrocarbons or PAHs, also increase the risk for PDA [48]. Whether alcohol consumption could be an independent risk factor of pancreatic cancer is still controversial. Yet frequent and excessive alcohol consumption is a cause of chronic pancreatitis, which serves as precursor for cancer. Chronic pancreatitis, diabetes and obesity are well accepted medical risk factors for pancreatic cancer.

1.2.2 Cancer evolution

Briefly, three stages are included in the evolution of pancreatic cancer [49]. Stage 1, risk factors of pancreatic cancer which have been listed before have impact on pancreas cells, a 'driver' mutation occurs which induces cells to escape from apoptosis and senescence or to survive under immune-surveillance. As shown in **Figure 3**, it takes around 11.7±3.1 years to form the non-metastatic parental cell clone with driver mutations. Stage 2, clonal expansion (stepwise or punctuated progression model) which implies that co-mutations would result in genomic instability and heterogeneity of tumor lesions along with the appearance of 'mixed' metastatic cell sub-clones, 63 somatic mutations could be found per tumor lesion in PDA [50]. It takes around 6.8±3.4 years for this stage. Stage 3, introduction to foreign microenvironments and index lesion formation. End stage of the disease with metastasis is approximately around 2.7±1.2 years.
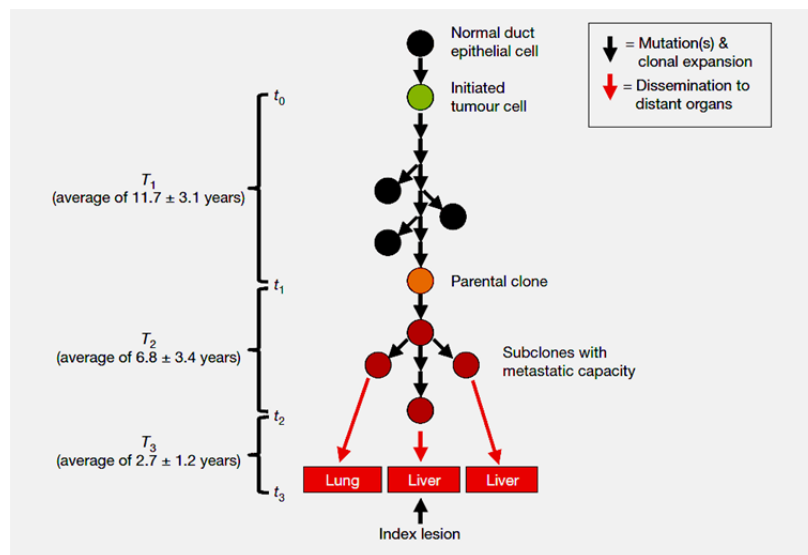

Figure 3. Genetic evolution of pancreatic cancer. ***Source: Distant metastasis occurs late during the genetic evolution of pancreatic cancer. Nature. 2010 Oct 28;467(7319):1114-7.***

1.2.3 Genomic instability and proteomic signatures

Signature mutations, included in the molecular profile of pancreatic cancer, are Kras, CDKN2A/P16, T53 and DPC4/SMAD4 [51]. Kras, located on chromosome 12, is a member of GTP binding proteins with intrinsic GTPase activity that transduces cellular proliferation, survival and differentiation signals [52]. Kras is detectable in almost all PDA cases and serves as a critical driver mutation. Cyclin dependent kinase inhibitor 2A (CDKN2A), located on chromosome 9, acts as a tumor suppressor gene, by inhibiting CDK4/6 dependent phosphorylation of the RB protein. CDKN2A inactivation could be found in 95% of PDA cases [53, 54]. CDKN2A is also involved in familial pancreatic cancer cases, known as familial atypical multiple-mole melanoma (FAMMM) syndrome. Patients are at risk of developing melanoma and exhibit a 12-22 fold increased risk to develop pancreatic cancer [55]. Located on chromosome 17, the tumor suppressor gene TP53 which is functional in cell cycle progression inhibition and DNA repair, is inactivated in 50-75% of PDA cases [53]. Deleted in pancreatic carcinoma, locus 4 (DPC4), located on chromosome 18, encodes a critical protein for TGF-β mediated growth inhibitory pathway, it is inactivated in 55% of PDA cases [55]. The most clinically relevant tumor marker for pancreatic cancer is CA19-9 with a specificity of 90% and sensitivity of 70-90%, which is also meaningful in treatment assessment and prognosis [56]. Other proteomic signatures include CA50, cancer embryotic antigen (CEA), CA72-4, osteopontin, and the regenerating islet-derived protein 4 (REG4) [57].

1.3 CANCER IMMUNOLOGY

1.3.1 Tumor immunogenicity

What represents the ideal target or a desired profile of a successful immunotherapy against cancer? Similar to infectious diseases, immunogenic responses should be long-lived which ensures that tumor cells could be recognized and then eliminated mainly by T cell responses. Tumor immunogenicity refers to the susceptibility of being targeted by the host immune response, and it may be associated with the antigenicity of tumor cell itself or from immunomodulatory products which are produced by the host or the tumor cells in the tumor microenvironment.

Tumor antigens are processed and presented as epitopes on major histocompatibility complex (MHC) class I or II molecules which could be recognized by T cells in the form of the MHC-peptide-TCR complex. Expression of MHC-I molecules is detectable on almost all nucleated cells, including tumor cells, which can be targeted by CD8+ T cells. MHC-II molecules are expressed on professional antigen-presenting cells (APCs) such as dendritic cells, macrophages, or B cells, which could be recognized by CD4+ T cells. MHC class II molecules could also be induced by IFN-γ on professional APCs. Several possibilities are available for the identification of tumor antigens. Expression cloning refers transfecting the tumor cDNA library into cells, followed by the ability of transfected cells in activating T-cell clones, gene-encoded peptides would be further tested for MHC affinity and target cell recognition [58]. Serological Analysis of cDNA expression library (SEREX) is a similar approach with the difference that the responsible gene encoding tumor antigen was defined via serum identification [59]. Another promising, but technical demanding, represents mass spectrometry. After immune-purification of MHC-I molecules together with the loaded peptides, peptides are eluted and then sequenced [60]. A new approach, reported by the group of Steven Rosenberg in 2014, a patient specific mutation database established by sequencing of the entire exons of the tumor; the identified peptides would undergo MHC molecule binding prediction and a tandem mini-gene would be employed in epitope exploration and to test whether the target is naturally processed and presented to T cells [61].

Based on the specificity and origin, TAAs could be divided into two groups and five subgroups (**Figure 4**).
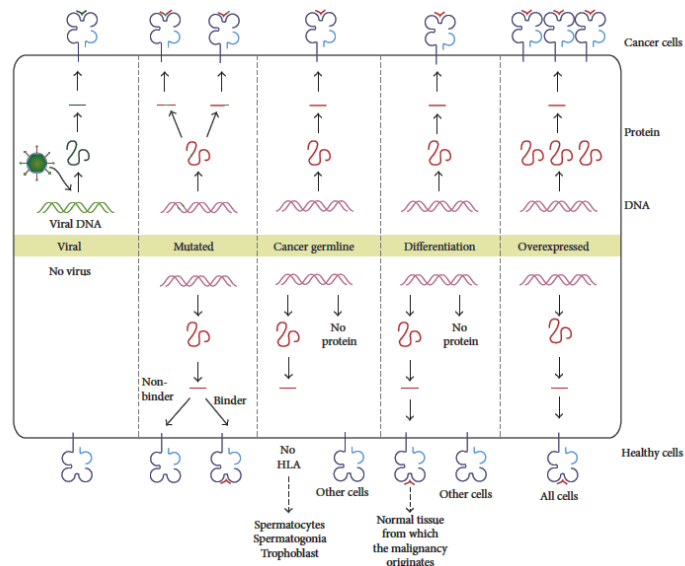
Figure 4. Tumor antigens. *Source. Human Tumor Antigens and Cancer Immunotherapy. BioMed research international, 2015: p. 948501.*

Antigens with high tumor specificity could be derived from viral proteins, mutations or germline line encoded proteins. A different group represents antigens with low tumor specificity, which includes differentiations or 'overexpressed' antigens. Around 15-20% of all human cancers are virus infection related [62]. A virus that could cause cancer, such as Kaposi's sarcoma-associated herpesvirus (KSHV), Hepatitis C virus (HCV), Epstein–Barr virus (EBV), Hepatitis B virus (HBV), Human papillomavirus (HPV), is called 'onco-virus'. An HPV vaccine which could induce HPV-16 specific immune response is now well accepted as a protection of HPV infection [63]. Therapeutic HPV vaccinations have also been successfully performed using 'long peptides' [63] and HPV-specific TCRs are used at the NIH (NCT02280811). Tumor mutations happen frequently, but not all mutations are involved in tumorgenesis, many of them emerge randomly such as mutations associated with cancerogens or radiation called 'passenger mutations', KRAS, EGFRvIII, TP53, IDH or CDKN2A mutations are called driver mutations and play a role in malignant transformation. Products from both conditions would be classified as TAAs. Cancer germline genes encoded proteins are another important source of TAAs. Located on X chromosome, the cancer germline genes could be expressed normally on trophoblastic or germline cells, or on tumor cells, known as cancer-testis antigens (CTAs). Gene products such as NY-ESO-1, MAGE, BAGE, GAGE belong to this group [64]. Differentiation antigens, such as CEA, gp100 or Melan-A/ MART-1 refer to antigens which could be expressed either in a given type of tumor or normal corresponding tissue. Similarly, overexpressed antigens such as WT1, HER2 or mesothelin could exist within healthy tissue, but increased expressions are detectable within certain tumor types.

1.3.2 Immunosurveillance

Immuno-surveillance refers to the function of the immune system that keeps foreign pathogens and malignant transformation 'in check'. Paul Ehrlich is perhaps the first person who introduced the concept of cancer immunosurveillance in 1909. He predicted that cancer would occur frequently if host defenses would not prevent the outgrowth of continuously arising cancer cells. The concept of immunosurveillance was first raised by Burnet and Thomas in 1957. Discovery of immune surveillance of tumors was proved in a mouse model by tumor transplantation experiments using syngeneic mouse strains. Increased incidence of EBV+ B cell lymphomas in transplant patients treated with immunosuppressive drugs and increased incidence of Kaposi's sarcoma and EBV+ B cell lymphomas in AIDS patients also serve as evidence for immunosurveillance in humans. One may argue that cancers, which occur spontaneously within the immune-competent population, are not under surveillance of the host immune system. Furthermore, many cancers may develop since the host immune system fails to discover them in situ, this will refers to the concept of 'tumor immune escape'.

1.3.3 Tumor microenvironment

If a tumor is considered as a 'malformed organ' instead of a random collection of malignant transformed cells, one would predict that tumor cells may even exceed healthy tissue in complexity and efficiency. Based on this observation, the concept of the 'tumor microenvironment' which reflects the crosstalk between malignant and non-transformed cells, as well as the dynamic network of cytokine is proposed. It was discussed that even the non-transformed cells, supporting malignant cells, should be also targeted in immunotherapy to achieve tumor clearance, as shown in preclinical animal models [65]. Undisputedly, cancer cells orchestrate and establish the complex environment via recruiting and instructing non-transformed cells localized or circulated to serve as supporting members. By physical contact or via the cytokine network, tumor cells specialize in 'convincing' and 'taming' normal cells into their 'accomplice'. Apart from tumor cells within tumor microenvironment, constituents of cancer stromal includes the tumor related vasculature and lymphatics, tumor infiltrating immune cells, cancer-associated fibroblasts, as well as pericytes and sometimes adipocytes [66] (**Figure 5**).

Cytokines such as VEGF, EGF, platelet-derived growth factor (PDGF)，fibroblast growth factor (FGF) and chemokines produced by tumor cells, or other stromal component could

keep the 'angiogenic switch' on, which means that endothelial cells could establish neo-vasculature with supporting pericytes [67], abnormal in both structure and function. For instance, heterogeneous neo-vessels could be found with uneven lumen, incomplete vessel wall and chaotic branching structures inducing leakage, uneven distribution of oxygen and nutrition [68]. A hyper-proliferative state of the tumor lesion could be impaired via inhibition of angiogenesis, which has already been used in clinical trials [67]. Immune cells infiltrating locally are either inhibited or silenced in function, or they act as tumor supporters within the tumor microenvironment. Beside tumor cells, immune cells are the main source of immune-suppressive cytokine like IL-10 or TGF-β. Other tumor promoting cytokines, induce mitogenic growth mediators which could stimulate proliferation of neoplastic cells and production of proteolytic enzymes, which could induce the modification of extracellular matrix (ECM) [69]. Within the tumor lesion, CD8+CD45RA- T cells and TH1 polarized inflammation, characterized by IL-2 and IFN-γ production, are associated with a better prognosis, while Treg (CD25highFOXP3+) cells, TH17 cells or TH2 polarized inflammation are linked with tumor progression [70]. γσ T cells are potent in anti-cancer activity, even against cancer stem cells. However, the linkage of the presence of γσ T-cells and prognosis in cancer patients is less clear [71]. Nature killer (NK) cells or NKT cells, which are innate cytotoxic lymphocytes, predict a better prognosis in certain cancer histology, but they appear not to exert killing in most cases, due to the suppressive tumor microenvironment [66]. Tumor infiltrating B-cells which could be found in draining lymph nodes and tumor invasive margins are reported with good prognosis only in several certain cancers and could play an role in tumor-promoting [72]. Clinical evidence showed that abundance of macrophages within tumor lesion is associated with poor prognosis. An unbalanced M1 macrophages (defined by production of IL-1, IL-12, IL-23 and chemokines) and M2 macrophages (defined by production of IL-10 and TGF-β) within the tumor is usually the case, where the M2 dominant environment could induce angiogenesis and immune-suppression [73]. Myeloid-derived suppressor cells (MDSCs) are defined as inhibitory immune cells, which could induce M2 polarization, Treg development and CD8+T cell inhibition [74]. Dendritic cells (DCs), professional APCs, are either defective or exist with impaired functions in the tumor micro-environment. They may not be able to immune-responses, or even worse, DCs have been reported to function as T cell suppressors by loading with 'suppressive epitope' (presented to T cells) or by activating Tregs. Cancer-associated fibroblast cells (CAFs) encompass distinctive cell types, such as activated connective tissue fibroblasts, proximal to tumor lesions, or myofibroblasts derived from mesenchymal stem cells (MSCs), either local or bone marrow orginated [75]. CAFs are significant in tumor niche formation and could

produce tumor-promoting growth factors like insulin-like growth factor1 (IGF1), hepatocyte growth factor (HGF) and FGF. The epithelial-mesenchymal transition (EMT), which is essential for tumor microenvironment building and metastasis induced by TGF-β, is positively affected by enzymes, produced by CAFs [76].
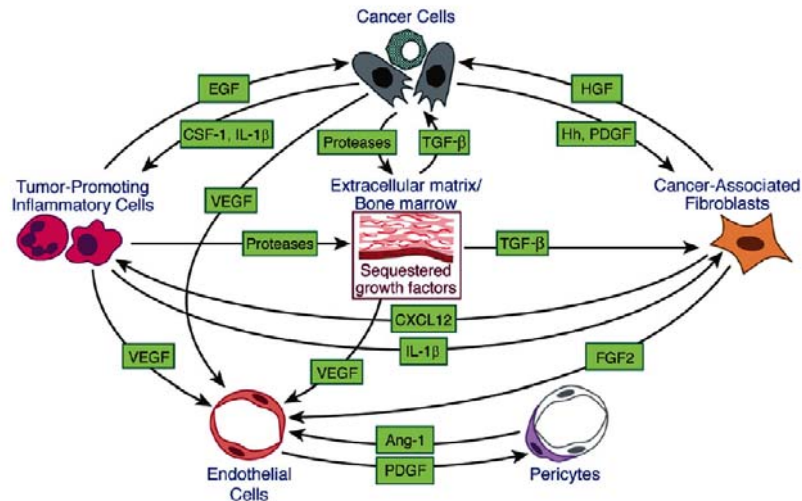
.

Figure 5. Tumor microenvironment with cytokine network. **Source:** *Hallmarks of cancer: the next generation. Cell, 2011. 144(5): p. 646-74.*

1.3.4 Tumor immune escape in glioma and pancreatic cancer

Different types of mechanisms have been demonstrated in immune escape of cancer cells. Tumor cell can simply 'outpace' the immune response by fast proliferation, or the tumor cells hide in immune-privileged sites. Loss of T cell recognition could be caused by: (1). Mutation or down regulation of tumor antigens [77]. (2). Down modulation and reduced expression of MHC-I and/or MHC-II molecules on tumor cells [77], (3). Loss of transporter associated with antigen processing (TAPs), low molecular mass poly-peptide (LMP) or other molecules involved with antigen processing [78]. (4). The tumor may generate intrinsic resistance to apoptosis by over-expression of bcl-2 [79], bcl-xL [80] or other inhibitors of apoptosis proteins (IAPs). (5). Frequency of Tregs may also increase within the tumor microenvironment and cause the tumor to facilitate generation of regulatory T cells [81]. Amino acid depletion derived metabolic immunosuppression could be another mechanism, ie. Arginine depletion would lead to down modulation of the TCR ζ chain or NF-κB, leading to defect activity of T cells and subsequent suppressed immune effective functions [82]. Tumor or tumor recruited Tregs may also produce inhibitory cytokines like IL-10 or TGF-β

[83]. Several malignancies, including neuroblastomas could up-regulate expression of macrophage migration inhibitory factor (MIF), inducing T cell activation blockage and T cell apoptosis [84]. Tumor cells could also express Fas ligand (FasL) which would induce apoptosis of T-cells. Up-regulation of ligands, like PD-L1 on tumor cells, receptor-binding cancer antigen expressed on SiSo cells (RCAS1) or CD200, which will bind to negative regulatory receptors on T cells may also lead to T cell suppression [85].

The brain was previously thought as 'immune privileged 'site, due to the existence of the blood-brain barrier (BBB). It is now well accepted that the brain has a well established immune network with a frequent crosstalk with the bodies' immune system. Microglia, act as the localized 'troops of immune system' within the brain, it keeps potential pathogens in check and removes the neurotoxic debris via phagocytosis. Structurally high vascularity areas, like the choroid plexus, the leptomeninges or circumventricular organs, which lack the BBB, could facilitate the exchange of proteins and cells [86]. The adaptive immunity was before considered to be limited within the brain due to the absence of lymphatic channels. The existence of functional lymphatic vessels, lining in the dural sinuses and connecting to deep cervical lymph nodes was reported as new channels for immune cell communication [87]. Even further, the disrupted BBB will allow immune cell infiltration in the case of glioma development.

Mechanisms employed in glioma immuno-escape are numerous. Despite the physical barrier of the BBB, microglia that may account for up to 30% of the glioma tumor mass, along with the tumor microenvironment, may produce cytokines including IL-6, IL-10, TGF-β, Prostaglandin E2 (PGE2), IL-1, CSF-1, MIC-1,EGF, Matrix Metalloproteinases (MMP), VEGF that suppress immune effector cells, promote tumorigenesis, induce M2 polarization and promote tumor invasion and migration [88-94]. CD70, gangliosides,PD-L1, CTLA4, CCL22, CCL2 and FasL expressed by GBM cells would induce apoptosis of cytotoxic T cells and attracts Tregs to the tumor site [95-97]. Similarly, pancreatic cancer also induces immune escape in the form of a suppressive microenvironment and suppressive cytokine networks [98-100].

1.4 IMMUNOTHERAPY FOR GLIOMA AND PANCREATIC CANCER

1.4.1 Non-targeted treatment

Current conventional standard treatment modalities for glioma include surgery, chemotherapy and radiation, while radiotherapy for pancreatic cancer is rarely applied. A first line chemotherapeutic agent for GBM is temozolomide which could increase the median and 2 year survival of patients with GBM by 2.5 months and 16.1% respectively [101]. Temozolomide is an oral drug that induces methylation of DNA at guanine residues of O6 or N7 position, such a methylation would induce DNA damage and cell death. Notably, some tumors with un-methylated promotor region of MGMT would be able to generate activated MGMT, which could repair the methylation damage induced by temozolomide; this is linked with poor prognosis [102]. Another combination drug for glioma treatment is lomustine which is an alkylating nitrosourea compound, that can cross the BBB easily [103]. Comparably, gemcitabine is the first consideration in PDA chemotherapy. As a difluorinated analog, gemcitabine blocks DNA replication in tumor cells which is related with treatment benefits in patients with PDA [104]. Even compared with 5-flluorouracil (5-Fu), which is another extensively used first-line anti-PDA drugs, gemcitabine showed its clinical survival in improving overall survival (OS) [105]. Gemcitabine based combination such as capecitabine, cisplatin or oxaliplatin in distinct clinical trials are explored and evaluated [106].

1.4.2 Monoclonal antibodies (mAbs)

Monoclonal antibodies (mAbs) may recognize TAAs in a MHC unrestricted manner and induce tumor cell death in various ways. Since glioma is defined as tumor with high vascularity, mAbs which target angiogenesis, such as VEGF as a monotherapy or in combination with other drugs, could achieve promising outcomes. Bevacizumab/Avastin (anti-VEGF mAb) binds to VEGF and neutralizes its biological activity so the downstream angiogenesis signaling is blocked [107]. EGFRvIII is detected in around 20-30% of GBM cases and involved in tumorigenesis and induction of chemotherapy resistance [108]. Cetuximab, a recombinant chimeric mAb with EGFRvIII specificity, is engaged in the EGFRvIII signal pathway with encouraging clinical results [109]. AMG595, an anti-EGFRvIII mAb and cytotoxic agent conjugated drug, is currently tested in clinical trials (NCT01475006). Similarly, Cetuximab is also involved in clinical trials for advanced PDA,

due to the key role of EGFR signal pathway in PDA pathogenesis. EGFR is overexpressed in 90% of pancreatic cancer cases and could serve as an immuno-target. Erlotinib, an anti-EGFR mAb, together with gemcitabine showed a reliable clinical improvement in one phase III trial [110]. Other mAbs like ganitumab or dalotuzumab which are specific to insulin-like growth factor (IGFR) have also been employed in pancreatic cancer therapy. Certain groups of mAbs, like checkpoint inhibitors, will be discussed below.

1.4.3 Vaccine based therapy

The aim of vaccine therapy is to induce and harness host adaptive immune response to tumor antigens actively via cell-based or non-cell based approaches. Anti-cancer directed immune response could be established de novo or by boosting pre-existent tumor specific memory cells. Potential antigens employed in cancer vaccine therapy may include: TAAs peptides or loaded to APCs, tumor cells with or without genetic modification, e.g. plasma DNA or vectors which encode TAAs. Vaccines targeted only a single tumor antigen appears to be associated with immune escape inevitably, so at least two or more antigens may be necessary. In order to broaden anti-tumor activity and to reduce tumor escape, immunoadjuvants are usually included, such as poly-ICLC which are stable dsRNAs and potent in IFN-γ induction. IMA-950, consists of 11 glioma tumor specific antigens, mainly with HLA-A2 and HLA-DRB1 restriction,IMA950 is a peptide-based vaccine, in combination with other drugs, or adjuvants that is broadly employed in different clinical trials in GBM vaccination [111]. SL-701, a combination of tumor antigens (IL13Rα2, HER2, gp100, MAGE-1 AIM-2) which are expressed in 75% of HLA-A1/2 glioma samples, has been tested with safety and tolerability outcome and showed clinical benefit [112]. Kras as a driver mutation in pancreatic cancer, was first tested in a peptide vaccine and 40% of patients showed prolonged survival in a phase I/II study [113]. Other tumor antigens like telomerase, gastrin or heat shock protein (HSP) also served as targets in a peptide-based vaccine against pancreatic cancer. HSP is generated under stress environment, such as inflammation or hypoxia. Similar to peptide-based vaccines, proteins such as HSP or CMV pp65 are involved in certain trials for glioma vaccination [114, 115]. Tumor cells could be candidates for vaccines, either directly as whole tumor cells or loaded onto dendritic cells as tumor lysates. In the case of glioma, autologous tumor cells with Newcastle disease virus (NDV) have been reported as vaccines [116]. This virus could selectively replicate in tumor cells to start an immune reaction in situ. Applied for pancreatic cancer, the GVAX pancreas vaccine is a whole tumor cell vaccine established with

a gene modified allogeneic pancreatic cell line with GM-CSF producing capacity [117]. Autologous dendritic cells (DCs) are the most commonly used APCs as a tumor vaccine due to their distinguished ability in activing the innate and the adaptive immune system. Glioma specific antigens such as EGFRvIII, CD133, HSP, SL701, IMA950 or glioma tumor lysates could be loaded onto autologous DCs or other APCs directly or through an intrinsic process (gene modified) and then recognized by the host adaptive immunity.

1.4.4 Checkpoints Inhibitors

Immune checkpoint mediators are to prevent excessive activation of immune system and to restrict immune response within to minimize the risk of autoimmune reactions. Pathological processes, like tumors, take advantage of this protective mechanisms and induce an immunosuppressive environment via those inhibitor checkpoint mediators, like cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) or the programmed cell death protein 1 (PD-1) to drive tumor specific effector cells into exhaustion or 'anergy'. In this case, therapeutic blockage of these inhibitory mediators would be linked to anti-tumor benefits which had been demonstrated in several cancers [118, 119]. CTLA-4 is expressed as a 'late co-stimulator' inducer to attenuate T cell activation and to induce memory T cell generation. Monoclonal antibodies against CTLA-4, like Ipilimumab, could inactivate signals downstream of CTLA-4 and reverse the destiny of tumor specific activated T cells. PD-1 expressed by antigen experienced T cells, could induce anti-tumor responses and also possibly autoimmune diseases. The PD-1 ligand could be expressed on tumor cells or tumor helper cells which would then cause T cell death by binding with PD-1 molecules. mAbs which could block PD-1 like Nivolumab, Pembrolizumab or PD-L1/2 like duravalumab, may rescue TAA-specific T-cells from apoptosis.

1.4.5 Adoptive cellular immunotherapy (ACT)

Adoptive cellular immunotherapy (ACT) has been first reported in 1990 by Kolb et al. after the treatment of chronic myeloid leukemia patients using donor leukocyte infusions (DLI) [120]. In 2002, Steven A. Rosenberg (NIH, DC, USA), reported that the passive transfer of highly specific tumor infiltrating T cells (TILs) directed against tumor differentiation antigens (i.e. Melan-A/MART-1, gp100) leads to durable regression in patients with metastatic melanoma. TILs showed a restricted TCR repertoire – which was also detectable after passive

TIL transfer in lympho-depleted hosts. The transfer and proliferation of T cells resulted in the regression of the patients' metastatic melanoma [121]. Genetically engineered lymphocytes induced cancer regression in patients has also been reported by Steven Rosenberg et al. in 2001 [122]. Here, MHC-class I and peptide-specific monoclonal TCRs were genetically expressed in recipient target cells which acquired the specificity of the transferred TCR. An 'immune escape' mechanism is the lack of a sufficient TCR repertoire capable of effectively recognizing tumor cells, a concept that has been called 'tumor – editing': long-term infection or tumor cells shape the TCR repertoire and lead to preferential expansion of T-cells that could potentially favor the life-cycle of the pathogen or proliferation of tumor cells (e.g. by production of growth factors). Alternatively, antigen-specific T-cells may exist, yet they are non-functional, they exhibit 'anergy' [123-125]. Therefore, functional deficiencies of antigen-specific cells, as well as a quantitative lack of antigen-specific T-cells in patients with infections (or tumors) call for a passive transfer of a T-cell product with a specific TCR that targets the nominal MHC class I/peptide complex on infected or transformed target cells (e.g. EBV, CMV, Adenovirus or tumor associated antigens, i.e. NY-ESO-1, EGFRvIII which are frequently expressed in glioma).

T cells are specialized in target specificity and functional tumor killing. In certain conditions, it may even negotiable to sacrifice the functional part of an organ ('disposable tissue') and pay attention only to the cancer specificity: one could regenerate genetic modified effector cells via TCR transfection. Similarly, T-cells can be endowed with chimeric antigen receptors (CARs) One potential advantage of ACT when comparing with vaccine-based strategies: the former is less limited by the clinical states of the patients, particularly when patients are immune compromised. T cells from several sources could be employed in ACT: TILs, PBMCs or engineered T cells. TILs are highly tumor specific, but TILs may be assimilated and inhibited by the tumor microenvironment, while the subdued state of TILs may be overcome during the ex vivo expansion with cytokines. Expanded TILs could be infused back for ACT as shown with clinical benefits in patient with melanomas [121]. TILs expanded with IL-2 were infused to 6 patients with recurrent malignant glioma in 1999. One patient showed complete response with 45month follow-up, and two achieved to partial response [126]. Re-infusing of enriched TILs from pancreatic cancer tumor tissue is tested in one ongoing phase II study (NCT01174121). Even though PBMCs are easier manipulated to meet the clinical requirement, the challenge is to enrich tumor specific T-cells. TCR transfection is one of the choices. For achieving this goal, Ag-specific T-cell clones are needed as the primary source for the specific TCR genes which can be transferred into target

cells, usually using a retroviral or lentiviral transfer system. Some tumor Ag-specific T-cells clones have already been established and tested in phase I clinical trials, as MAGEA3 HLA-A*0101-EVDPIGHLY, NY-ESO HLA-A*0201-SLLMWITQC, the MAGE-1 HLA-Cw*1601- SAYGEPRKL restricted T cell clone or the Kras G12D HLA-C*08:02 specific T cell clone[127]; the TCR gene cDNA sequences were isolated from the T-cells clones, after initial functional testing and validation, followed by creation of a retroviral vector which could encode a T cell receptor (TCR) specific for the nominal target antigen. Genetically fusing extracellular binding domains such as tumor antigen specific IgG with an intrinsic signaling domain could generate effector CAR T cells, which recognize antigens in a MHC independent way. CD133, EGFRvIII, IL13Rα2 or HER2 specific CAR T cells are undergoing clinical exploration in a series of clinical trials (NCT01109095, NCT00730613, NCT01082926, NCT01454596). In pancreatic cancer, genetic modified TCR or CAR T cells are targeting mesothelin or survivin in different trials (NCT01583686, NCT01967823, NCT02239861).

2 AIMS OF THE THESIS

The general aim of this thesis is to expand functional tumor specific T cells from peripheral blood or tumor section/biopsy of patients with glioma or pancreatic cancer which could be potentially be used for cellular immunotherapy. This would enable that TAAs-specific TCRs could be isolated and sequenced for further potential clinical use. We also attempted to understand relations between peripheral blood immune reactions against tumor antigens as predictors and patients overall survival (OS).

Specific aims

- To expand Tumor infiltrating lymphocytes (TILs) from fresh tumor tissues using IL-2, IL-15, IL-21 to large scale. Tumor tissues are received from surgical resections or from biopsy of patients with glioma or pancreatic cancer. The TIL specificity, clonality and function will be defined. (Paper I and II)

- To define expression of NY-ESO-1 and survivin in glioma tumor lesions and relevant T cell response in peripheral blood immunoreaction together with further attempts to expand functional NY-ESO-1 or survivin specific T cells from autologous PBMCs or TILs of patients with glioma. (Paper III)

- To define expression of mesothelin in glioma tumor lesions and relevant ex vivo peripheral blood immuno-reactions. (Paper IV)

- To define ex vivo peripheral blood immunoreaction against a list of viral and tumor antigens in patients, with glioma, to explore linkage of antigen immune responses with patients overall survival (OS). (Paper V)

3 RESULTS AND DISCUSSION

3.1 Tumor-infiltrating lymphocytes (TILs) from patients with glioma or pancreatic cancer (Paper I and II)

TILs could reliably be expanded from surgical resections or biopsy specimens from 17 patients with pancreas cancer and 16 patients with glioma up to 10^{10} cells using the IL-2/IL-15/IL-21 cytokine cocktail with a dominant CD3+ phenotype. The benefit of combining IL-2, IL-15 and IL-21 in TIL expansion could be due to several factors, e.g. IL-21 has been shown to promote expansion of TILs with strong cytotoxic potential [128], it rescues CD8+ T-cells from suboptimal antigenic stimulation and it has been shown to stimulate high affinity T-cells without the need for CD8 help [129]; IL-15 and IL-21 may therefore aid to expand 'better' T-cells with increased frequencies of antigen-specific responses residing in long-term memory T-cell subsets [130]. The preferential expansion of central memory and effector T-cell subsets, defined by CD45RA-CCR7+ and CD45RA-CCR7- expression in both glioma and pancreatic cancer cases, appears to be associated with the nature of T cell source, since IL-21 may have minimal effects on 'resting' cells but could selectively expand T cell in the activation state. TILs with certain characteristics which refer to distinct effector functions and homing patterns are necessary for clinical responses [131]: Central memory T-cells (CD45RA-CCR7+) have been linked with stronger proliferative potential and are the best candidates to provide potential long-term anti-tumor protection [132, 133]. Central memory T cells are reported to relate with better prognosis and long-term (up to 3 years) remissions in some cases [134].

TILs were further characterized for expression of "activation-exhaustion" cell surface markers exhibiting a low median frequency of 4-1BB, CTLA-4, LAG-3 and TIM3 positive cells in CD3+ TILs, whereas PD-1+ T-cells could be an indication of tumor antigen experienced cells that could potentially be further expanded ex vivo. TILs with Treg phenotype (CD3+CD4+CD25highCD127-Foxp 3+) are not detectable in both tumor histologies.

The function of T cells refers to cytotoxicity and/or cytokines like IFN-γ, TNF-α and IL-2 against autologous tumor or TAAs. Cytotoxicity can be tested via CD107a Assay or the Cr51 release assay, while cytokines could be measured by enzyme-linked immunosorbent assay

(ELISA) or intracellular cytokine staining (ICS). TILs expanded either from glioma or pancreatic cancer samples have been shown to react to autologous tumor cells defined by cytotoxicity and cytokine production. TILs have also be shown to react to TAAs i.e. mesothelin, NY-ESO-1 and survivin (**Figure 6**). Both tumor specific cytotoxicity and cytokine productions could be blocked by anti-MHC-I/II Abs which means the tumor specific immune response are based on MHC class I/II- TCR interaction.
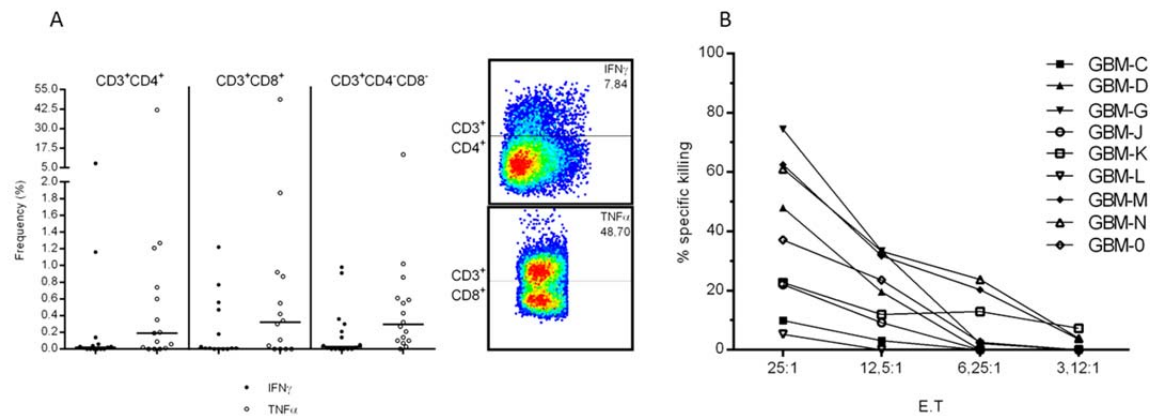

Figure 6. Immunoreaction of TILs from glioma against autologous tumor cells. A. Cytokine production of TILs in different subpopulations. B. Autologous tumor cell specific cytotoxicity of TILs defined by Cr51 release assay.

Clonality of TILs from either glioma or pancreatic cancer samples were defined via flow cytometry-based Vβ analysis and polymerase chain reaction (PCR)-based clonality analysis. Preferential expansion of certain TCR Vβ clones were detectable in both tumor origins (glioma and pancreatic cancer), Some TCR Vβ families were shown to monoclonal or oligclonal proved by PCR and DNA sequence. One dominant TCR Vβ monoclone, after expansion, was tested for reactivity with autologous tumor cells. (**Table 2**)

TIL id	Vβ	Vβ	(-D-)	Jβ	Jβ	Flow antibody
GBM A-CD4	TRBV20-1	CSA	ATGDRP	YEQYF	TRBJ2-7	Vβ2
GBM A-CD8	TRBV10-3	CAI	RTGSD	NEQSF	TRBJ2-1	Vβ12
GBM A-CD8	TRV11	CAS	RYTGS	IEQFF	TRBJ2-1	Vβ21.3
GBM F-CD4	TRBV27	CAS	SAGTSGVT	YEQYF	TRBJ2-7	Vβ14
GBM G-CD4	TRBV6-1/5/6	CAS	STR	FEQYF	TRBJ2-7	Vβ13.1
GBM H-CD8	TRBV12-3/4	CAS	SSRRDHTY	NGQFF	TRBJ2-1	Vβ8
GBM H-CD8	TRV27	CAS	SLQGAN	YGYTF	TRBJ1-2	Vβ14
GBM I-CD4	TRBV20-1	CSA	RVIPSGGVVQGT	DTQYF	TRBJ2-3	Vβ2
GBM I-CD8	TRBV5-1	CAS	SWDKS	YEQYF	TRBJ2-7	Vβ5.1
GBM J-CD4	TRBV11	CAS	SRLALFS	YEQYF	TRBJ2-7	Vβ21.3

Table 2. TILs Vβ sequence after preferential expansion.

3.2 NY-ESO-1-specific T-cell responses in PBMCs from patients with glioma (PAPER III)

Immunohistochemistry was used to test for survivin (n=40 samples) and NY-ESO-1 (n=38 samples) expression in tumor specimens. 50% of samples were defined as 'high' ($\geq$20%) expressing survivin and 39.4% of samples were NY-ESO-1 expression-positive ($\geq$5%)
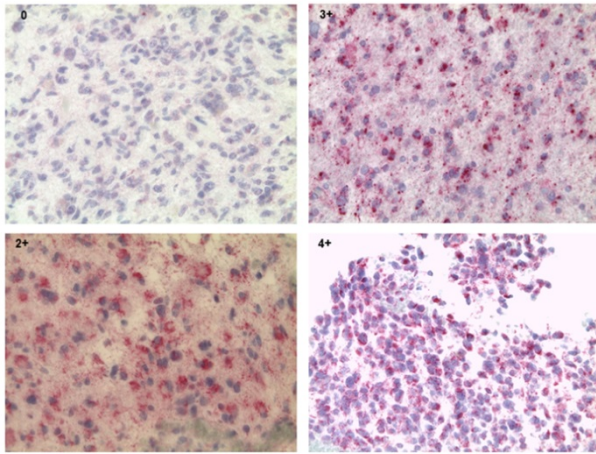

Figure 7. NY-ESO-1 immunohistochemistry

(**Figure 7**). NY-ESO-1 expression in glioma was found to be low in a single study [135] as compared to the current report. A possible explanation for this difference may be the selection of patients. The patients in our cohort were patients with a primary tumor who did not receive radiation or any prior chemotherapy [136]. NY-ESO-1 expression was also found to be patchy; limited access to tumor material may therefore result in false negative results concerning protein expression. NY-ESO-1 and survivin expression was consolidated by the presence of humoral anti-NY-ESO-1 and anti-survivin directed IgG responses [137, 138] in the patient cohort. NY-ESO-1 expression in glioma may open new therapeutic options, given the recent success of anti-NY-ESO-1 directed transgenic TCRs (for HLA-A2+) individuals [139, 140], the use of anti-NY-ESO-1 directed antibody therapies [141], or the use of anti-NY-ESO-1 directed vaccination strategies [142].

NY-ESO-1 and survivin were tested to drive cellular proliferation and IFN-γ production in blood from patients with glioma. We identified an association of tumor associated antigens (TAAs)-reactive T-cells (defined by IFN-γ production) in correlation with the histopathological grading of the tumor and T-cells cultured with IL-2/IL-15 and IL-21. Stronger IFN-γ production was identified in PBMCs from patients with histopathological grade III tumors as compared to patients with a grade IV tumor in response to NY-ESO-1 (p = 0.0135), as well to the survivin peptide mix (p = 0.0062, supplementary). The proliferation ratio was increased using IL-2/IL-15/IL-21 as compared to IL-2/IL-7 for NY-ESO-1 (p = 0.0014) driven T-cell expansion. The proliferative capacity of PBMCs in response to TAAs suggested that NY-ESO-1 or survivin directed T-cells can be expanded and may be used for

the cellular therapy of patients with glioma. Furthermore, NY-ESO-1 tetramer+ sorted or INF-γ captured NY-ESO-1 directed T-cells were shown to recognize naturally processed and presented NY-ESO-1 epitopes on glioma tumor cell lines suggesting that peptide-driven expansion of T-cells leads to biologically and clinically relevant T-cell populations directed against tumor cells (**Figure 8**).

In order to evaluate the cytokine production at a single cell level, we expanded PBMCs (after ficoll separation) from 5 patients with the NY-ESO-1 or the survivin peptide mix in the presence of IL-2/IL-15/IL-21, tested for T-cell maturation (based on CD45RA/CCR7 and T-cell activation markers, including 4-1BB. We found a trend of increase in central memory and effector memory T cells and also 4-1BB expression after expansion. Anti-NY-ESO-1 reactivity was confirmed by MHC-class I (HLA-A2+) –peptide-tetramer guided staining showing up to 9.25% HLA-A2+ (NY-ESO-1) reactive T-cells.
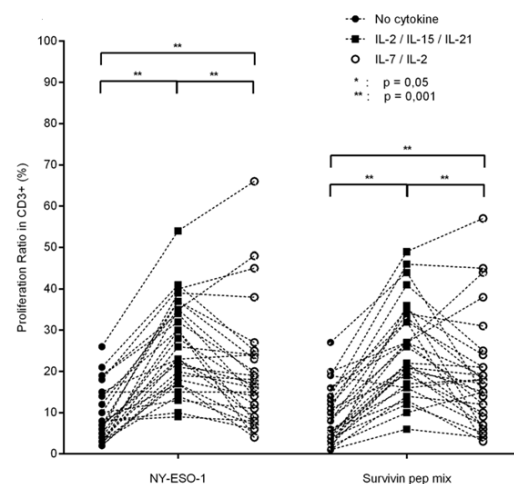

Figure 8. NY-ESO-1 and survivin induced cell proliferation.

The numbers of cellular therapies directed against tumor – associated antigens for patients with gliomas are limited up to now; a review of cell-based therapies suggests that infusion of immune cells may lead to improved survival along with limited therapy associated toxicity [126, 143-146]. For instance, PBMCs were harvested for cellular therapy and CTL were generated directed against autologous (glioma) tumor cells (using a mix of PBMCs, autologous tumor cells and recombinant IL-2), followed by in situ administration (10^8 up to 10^9 T-cells i.t.). 3/5 patients did not exhibit any benefit; 1/5 patient showed a transient regression and 1/5 a complete regression that lasted 104 weeks [147]. The data in our report show that NY-ESO-1 and survivin is can now be added as a tumor-specific target for the biological treatment of patients with glioma particularly since data from several NY-ESO-1 [7] or anti – survivin [148] directed trials did not suggest major toxicity.

3.3 Mesothelin as a novel biomarker and immunotherapeutic target in human glioblastoma multiforme (Paper IV)

Mesothelin is a 40 kDa tumor differentiation antigen present on normal mesothelial cells, but overexpressed in mesothelioma, meningioma, ovarian cancer, lung and pancreatic adenocarcinomas [10, 149]. Mesothelin expression is closely related with prognosis [150-154]. Full-length, unprocessed mesothelin precursor comprises two components, namely the 31 kDa megakaryocyte-promoting factor (MPF) [155] and the membrane-anchored, 40 kDa mesothelin-glycoinositolphoslipid (GPI) component. MPF is cleaved by furin and shed into systemic circulation, and has been evaluated as a more accurate biomarker as compared to the full-length mesothelin for the immunodiagnosis of mesothelioma. Cell membrane-bound (or mature) mesothelin selectively binds to mucin 16 (MUC16) [156], which is expressed in the peritoneum, pleural cavities, mucosal surfaces and the brain, and has been shown as a promising target in immunotherapy [157-160]. We confirmed the overexpression of mesothelin with ratio of 36.4% (4/11) in GBM tissue by immune-histological staining of mesothelin protein in paraffin-embedded tissue sections visualized via fluorescence microscopy (**Figure 9**)
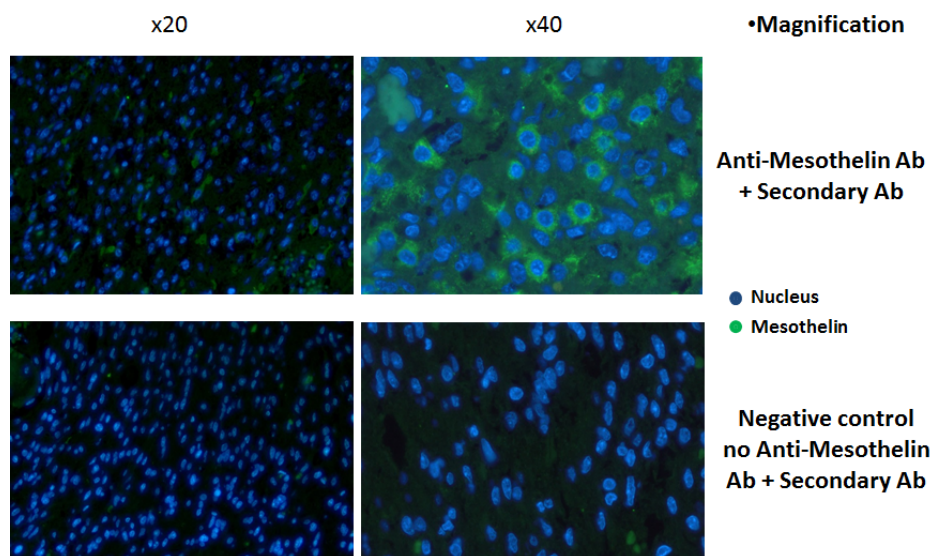

Figure 9. immune-histological staining of mesothelin

Mesothelin (mesothelin peptide pool (297-630aa)) was tested to drive IFN-γ production in blood from patients with brain cancer in different histology and grade. Within three conditions of co-incubation (no cytokine,IL-2/15/21 or IL-2/7), we found that conditioning of whole blood from GBM patients with IL-2, IL-15 and IL-21 significantly improved the IFN-γ response to the mesothelin peptide pool, as well as the MPF and mesothelin precursor

subcomponent. Conditioning of whole blood with IL-2 and IL-7 resulted in a stronger IFN-γ response to all three antigens (i.e. mesothelin precursor, MPF and mesothelin) although to a lesser degree than the combination of IL-2, IL-15 and IL-21. The finding was similar for patients with astrocytoma, oligoastrocytoma/ oligodendroglioma (OA/OD) and metastatic brain tumors with regard to IFN-γ response to full-length mesothelin and MPF with IL-2, IL-15 and IL-21 conditioning.

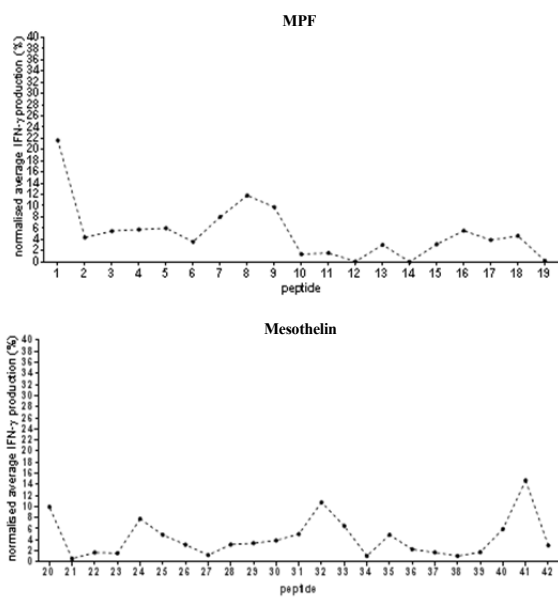After confirming that patients with brain cancer can mount measurable cellular immune responses to the mesothelin precursor, we wanted to ascertain which epitopes within the mesothelin protein evoke the strongest IFN-γ response by T cells. Briefly we used a pool of 42 chemically synthesized peptides spanning the full-length mesothelin. The first 18 peptides comprise the MPF component, while the following 24 peptides constitute the mature mesothelin domain. We plotted the absolute values for IFN-γ production per patient, as well as the percentage of normalised average response. (**Figure 10**) TILs generated from GBM patients, expanded with cocktail IL-2/15/21, were also evaluated for their response to mesothelin peptides and we observed anti-mesothelin reactive TILs defined by ICS (0.41% of IFN-γ and 0.71% of TNF-α in CD3+ T -cells)

Figure 10. Full length mesothelin mapping

To the best of our knowledge, mesothelin has not been studied in the context of malignant brain cancer in humans. Using immunofluorescence microscopy, we could visualize that the mesothelin protein is overexpressed in human GBM tissue samples. Furthermore, our immunological data suggests that T cells from patients with malignant primary glioma (i.e. GBM) can strongly recognize and respond to cell surface-bound mesothelin (GPI-anchored component) via cytokine production (IFN-γ and TNF-α). T cells from patients with GBM are able to expand strongly in the presence of conditioning of growth medium with IL-2/IL-15/IL-21 to the mesothelin peptide pool. This also applies to TILs from patients with GBM or pancreatic cancer, which are usually in contact with antigen-expressing cells in the tumor microenvironment [161].

3.4 Survivin peptide-specific cellular immune responses and cytokine networks predict the improved survival of patients with glioblastoma multiforme (Paper V)

Central nervous system (CNS) cancers exhibit a very poor prognosis in patients, although they are significantly less frequent than other solid tumors i.e. lung cancer, melanoma, pancreatic cancer etc. [162]. The most common and aggressive clinical manifestation of glioma is GBM, which presents a 5-year survival less than 4% [162] , compared to the other primary gliomas (WHO grade II and III), which exhit a 5-year survival rate of at least 50%. Patients (n=205) with the following diagnoses of malignant glioma were selected to participate in the study: glioblastoma multiforme (GBM, WHO grade IV CNS tumor, n=145) or non-GBM (n=60), comprising patients with astrocytoma, oligodendroglioma or not otherwise specified (NOS) categories, which may include oligoastrocytoma or anaplastic oligoastrocytoma (WHO grade II-III CNS tumors) [18]. Venous blood for laboratory studies was drawn from the participating patients for performance of the whole blood assay (WBA) and serum collection on the day of the surgery and prior to initiation of radio- and chemotherapy. Survival analysis in this paper focused on GBM and non-GBM cases that served as controls.

The univariate analysis (The Kaplan-Meier (K-M) survival analysis) with log-rank test was performed by comparing single parameters (demographic, clinical, immunological and antigen-specific immune response) with the overall survival of patients with GBM. Demographic and clinical factors found in univariate analysis include: age of patients (p=0.0439), tumor recurrence (p=0.0397), Karnofsky Performance Status (KPS) of patients (p=0.0258), recursive partitioning analysis (RPA) before surgery (p=0.0435), radiotherapy (p<0.0001) and chemotherapy (p<0.0001).

We found serum IL-4, IL-5 and IL-6 levels are significantly related between each two based on spearman correlation analysis (IL-4 vs IL-5, IL-5 vs IL-6 or IL-4 vs IL-6), similar to serum IFN-γ, TNF-α and IL-17A. When analyzing patients OS, we found that the entire set of IL-4, IL-5 and IL-6 as a pattern, i.e. either all three cytokines present or all absent ('all' or 'none') correlated with a better survival profile (p=0.0022) among the patients compared to only a 'partial' combination (e.g. IL-4 and IL-5 are detectable but no IL-6, or IL-5 is detectable but no IL-4 and IL-6). The scenario is similar for IFN-γ/TNF-α/IL-17A levels in

serum; patients with all of the cytokines or none of cytokines tend to exhibit an improved survival pattern (p=0.0235). (**Figure 11**)
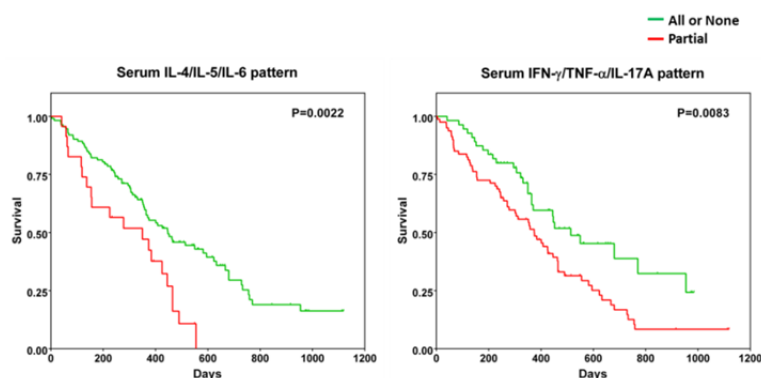

Figure 11. Serum cytokine pattern related with patients OS

We also measured antigen specific IFN-γ responses in peripheral blood from patients with GBM incubated with antigens (peptide mixes or single peptide): i.e. CMV pp65, EBV EBNA-1 and EBNA-3a, NY-ESO-1, survivin, mesothelin, EGFRvIII, survivin peptide 97-111 (TLGEFLKLDRERAKN), NY-ESO-180-94 (ARGPESRLLEFYLA) in three conditions (no cytokine, IL-2/15/21 or IL-2/7) and correlated the cytokine production with the patients OS. Factors which showed significant relation with OS included: IFN-γ responses against CMV Pp65, EBNA-1, EBNA-3a and survivin97-111 cultured with IL-2/15/21. No single factor was observed with significance in condition to 'no cytokine' or 'IL-2/7'.

The single factors we mentioned above (i.e. demographic, clinical, immunological and antigen-specific immune response) were significantly correlated with patients' OS in the univariate analysis (with a $P<0.05$ as cut-off), those parameters could be recruited into a Cox Proportional hazards model (forward and backward stepwise analysis) for multivariate analysis, results are showed in **Table 3**. Based on the COX hazards analysis, we identified the factors related with patients OS are: i). clinical parameters (chemotherapy and radiotherapy), ii). immunological parameters (serum IL-4/5/6 pattern and serum IFN-γ/TNF-α/IL-17A pattern) ,iii). antigen-specific immune response to survivin 97-111 and EBNA-1.

Stepwise(COX)	HR	P	95% CI	
Radiotherapy	0,3368	<0.0001	0,20435	0,55502
Chemotherapy	0,7143	0,028	0,52857	0,96521
EBNA1	1,6397	0,051	0,99820	2,69339
Survivin97-111	2,0756	0,024	1,09916	3,91960
IL4/5/6	1,7851	0,052	0,99582	3,19990
IFN-γ/TNF-α/IL-17A	2,2645	0,003	1,33067	3,85354

Table 3. COX analysis confirmed single factors to predict survival of patients with GBM

4 CONCLUSION

- 'Classic' TAAs such as NY-ESO-1, survivin and mesothelin are expressed in glioma tumor lesions.

- Autologous tumor cells, tumor- or viral- antigens (NY-ESO-1, survivin, mesothelin or CMV Pp65) specific T cells either from peripheral blood or tumor tissues could be successfully expanded (large scale) within 4 weeks in IL-2/IL-15 and IL-21 showing a Th1-cytokine production pattern. These antigen-specific T-cells exhibit cytotoxicity and/or cytokine production and represent an attractive profile for cellular immunotherapy.
- We found that serum cytokines like IL-4, IL-5, IL-6 in a combinational pattern and immunoreaction to survivin 97-111 or EBNA-1 could be employed as predictor of prognosis for patients with GBM. Survivin91-111 could serve as a target for ACT for patients with glioma.

5 FUTURE WORK

- We could establish a number of NY-ESO-1 and survivin specific T cell line(s) from PBMCs from patients with glioblastoma. These T-cells produced IFNγ and TNFα after expansion. According to our unpublished data, we were able to expand T-cells from 35 million to 2.2 billion within 3 weeks, a cell number which would meet the scale for clinical therapy requirement. We will focus to streamline the T-cell expansion process and to test whether sufficient numbers of T-cells could be expanded for future biological therapy, including the preferentially expansion of NY-ESO-1 or survivin directed T-cells.

- We could reliably expand TILs from brain tumor and pancreatic cancer tissues with strong reactivity and specificity to autologous tumor cells, the TILs showed potent cytokine production and cytotoxicity. We will focus on the further characterization of the TILs, including functional and phenotypical analysis and subsequent TCR sequencing in order to develop fast and effective T cells products which could be for potential products for T cell therapy.

- We had shown that the ex vivo expansion of TILs from patients with glioma and pancreatic cancer leads to strong expansion of certain TCR Vβ families, defined by flow cytometry. We postulate that these expanded TCR Vβ families are directed against the patient's own tumor cells. We plan therefore to sort these cells and perform TCR sequence analysis by PCR. Specificity could be tested for selected T-cell lines by TCR transfer; a similar approach would be used for the NY-ESO-1 tetramer sorted T cells in order to obtain a broader repertoire of anti-NY-ESO-1 directed TCRs that could be used for biological therapy.

- We plan to purify and expand mesothelin epitope specific cells from PBMCs and TILs of patients with glioma via mesothelin dextramer sorting for further TCR sequencing and TCR transfer.

- We plan to carry out mesothelin immunohistochemistry on glioma samples with different histology of glioma (grade I-IV) to study mesothelin expression in glioma with GBM histology.

- We plan to purify survivin 97-111 epitope directed T cells for TCR sequencing which could be useful for future treatment due to the correlation between survivin 97-111 immune responses and patient's survival.

6 ACKNOWLEDGEMENTS

Lalit, when I started in cell lab, it was you who taught me how to culture T cells and shared tons of knowledge with me in the area of immunology and immunotherapy. In my impression, you were the one so smart and always came with a lot of super cool ideas. Good luck for your research and life in Norway!

Thomas, in my mind, you are not only the one who sit in the corner with headphone, you taught me how to separate T cell from blood which was the first thing I learned in the very beginning and how to do ELISA. You showed me how to make figures for publication, and provide tremendous help in my manuscript writing. The basic lab and computer skills which I learned from you make me change from a medical doctor to a researcher. My friend, I am looking forward to meet you in China, then we could get drunk together!

Qingda, thank god to put you here in this group, your special attitude and points, your curiosity to almost everything, your great help in work and life, and important issue, we can discuss all kinds of things in Chinese, even though others may think we are fighting in Chinses. You are one of the most important friends in my life who keeps me from depression in the past 5 years.

Martin, working together for almost 4 years, thank you for all your help in my manuscript writing, thank you for sharing you knowledge with me. I must say, it is an enlighten experience to have discussion with you. My friend, no matter where you are in the future, I believe you will be someone in this area.

Rebecca, you are the senior researcher who still works in this group since I started here. Thank you for all your scientific advices and help. And also send my regards to your family and your kids.

David, beside the scientific advices, I would also appreciate the happiness you bring to us. Take care, my friend.

Marlene, for all the help with the administrative issue, for all the discussion and heart-warming words, I feel safe since I know you are standing behind and supporting us all the time.

Esther, I witness your improvement in lab work within one year. You are smart and efficient in knowledge learning and utilizing, you has a bright future for sure. As a friend, your happiness and cool attitude always influences me. Welcome to China, you can try thousands of different food and snacks there, even dumplings have more than hundred kinds of fillings. By the way, sometimes I eat your snacks on your desk without telling you, but not only me, qingda also did.

Aileen, congrats again, maybe I will forget the experiments we did together, but I will not forget we encourage each us in our tough times. Tough times never last but tough people do. Thank you for your company, my friend.

Rose, it is a talent that you can almost kill sadness and injuries by eating some fine food. I will not forget your sincere help. You will be a successfully business woman soon. Just don't forget me when you become a millionaire.

Anurupa, you are nice and considerable, you plan food and gift for other's birthday. You are responsible, you remind people with the routine lab work and cleaning, you do the kitchen duty for the group. For all your help, we keep in mind, thank you!

Georgia, you are working hard, you are the one who takes care of almost all the clinical samples nowadays. Take care!

Anna, even you only worked with us for couple of months, but you provide great help for my project, I had never see anyone before who could work as fast and accurate as you did. Just good luck and take care!

Moa, thank you for helping me to arrange my dissertation venue .

There are so many names come to my mind, my colleagues, my friends, my parents, my families… But I know I can't just express my appreciation by a simple and pale 'thank you', instead, I will your smiling faces in my memory until the end of my life. All the best, and just don't forget, you once have a friend from China.

7 REFERENCE

1. Bercovici, N., et al., *New methods for assessing T-cell responses.* Clinical and diagnostic laboratory immunology, 2000. **7**(6): p. 859-64.

2. Chang, K., et al., *Characterization of the antigen (CAK1) recognized by monoclonal antibody K1 present on ovarian cancers and normal mesothelium.* Cancer research, 1992. **52**(1): p. 181-6.

3. Taniguchi, Y., et al., *Mechanism for maintaining homeostasis in the immune system of the intestine.* Anticancer research, 2009. **29**(11): p. 4855-60.

4. Dunn, G.P., et al., *Cancer immunoediting: from immunosurveillance to tumor escape.* Nature immunology, 2002. **3**(11): p. 991-8.

5. Dunn, G.P., L.J. Old, and R.D. Schreiber, *The three Es of cancer immunoediting.* Annual review of immunology, 2004. **22**: p. 329-60.

6. Mamelak, A.N. and D.B. Jacoby, *Targeted delivery of antitumoral therapy to glioma and other malignancies with synthetic chlorotoxin (TM-601).* Expert opinion on drug delivery, 2007. **4**(2): p. 175-86.

7. Bielamowicz, K., S. Khawja, and N. Ahmed, *Adoptive cell therapies for glioblastoma.* Frontiers in oncology, 2013. **3**: p. 275.

8. Meng, Q., et al., *Expansion of Tumor-reactive T Cells From Patients With Pancreatic Cancer.* Journal of immunotherapy, 2016. **39**(2): p. 81-9.

9. Gnjatic, S., et al., *NY-ESO-1: review of an immunogenic tumor antigen.* Advances in cancer research, 2006. **95**: p. 1-30.

10. Soleimanpour, E. and E. Babaei, *Survivin as a Potential Target for Cancer Therapy.* Asian Pacific journal of cancer prevention : APJCP, 2015. **16**(15): p. 6187-91.

11. Widenmeyer, M., et al., *Promiscuous survivin peptide induces robust CD4+ T-cell responses in the majority of vaccinated cancer patients.* International journal of cancer, 2012. **131**(1): p. 140-9.

12. Hassan, R. and M. Ho, *Mesothelin targeted cancer immunotherapy.* European journal of cancer, 2008. **44**(1): p. 46-53.

13. Ostrom, Q.T., et al., *CBTRUS statistical report: Primary brain and central nervous system tumors diagnosed in the United States in 2006-2010.* Neuro-oncology, 2013. **15 Suppl 2**: p. ii1-56.

14. Grier, J.T. and T. Batchelor, *Low-grade gliomas in adults.* The oncologist, 2006. **11**(6): p. 681-93.

15. Ohgaki, H. and P. Kleihues, *Population-based studies on incidence, survival rates, and genetic alterations in astrocytic and oligodendroglial gliomas.* Journal of neuropathology and experimental neurology, 2005. **64**(6): p. 479-89.

16. Dang, L., et al., *Cancer-associated IDH1 mutations produce 2-hydroxyglutarate.* Nature, 2010. **465**(7300): p. 966.

17. Jiao, Y., et al., *Frequent ATRX, CIC, FUBP1 and IDH1 mutations refine the classification of malignant gliomas.* Oncotarget, 2012. **3**(7): p. 709-22.

18. Dunn, G.P., et al., *Emerging insights into the molecular and cellular basis of glioblastoma.* Genes & development, 2012. **26**(8): p. 756-84.

19. Killela, P.J., et al., *The genetic landscape of anaplastic astrocytoma.* Oncotarget, 2014. **5**(6): p. 1452-7.

20. Louis, D.N., et al., *The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary.* Acta neuropathologica, 2016. **131**(6): p. 803-20.

21. Ohgaki, H. and P. Kleihues, *Genetic pathways to primary and secondary glioblastoma.* The American journal of pathology, 2007. **170**(5): p. 1445-53.

22. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.

23. Dietrich, J., E.L. Diamond, and S. Kesari, *Glioma stem cell signaling: therapeutic opportunities and challenges.* Expert review of anticancer therapy, 2010. **10**(5): p. 709-22.

24. Lathia, J.D., et al., *Cancer stem cells in glioblastoma.* Genes & development, 2015. **29**(12): p. 1203-17.

25. Brescia, P., C. Richichi, and G. Pelicci, *Current strategies for identification of glioma stem cells: adequate or unsatisfactory?* Journal of oncology, 2012. **2012**: p. 376894.

26. Kemper, K., et al., *The AC133 epitope, but not the CD133 protein, is lost upon cancer stem cell differentiation.* Cancer research, 2010. **70**(2): p. 719-29.

27. Beier, D., et al., *CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles.* Cancer research, 2007. **67**(9): p. 4010-5.

28. Bonavia, R., et al., *Heterogeneity maintenance in glioblastoma: a social network.* Cancer research, 2011. **71**(12): p. 4055-60.

29. Wikstrand, C.J., S.H. Bigner, and D.D. Bigner, *Demonstration of complex antigenic heterogeneity in a human glioma cell line and eight derived clones by specific monoclonal antibodies.* Cancer research, 1983. **43**(7): p. 3327-34.

30. Jung, V., et al., *Evidence of focal genetic microheterogeneity in glioblastoma multiforme by area-specific CGH on microdissected tumor cells.* Journal of neuropathology and experimental neurology, 1999. **58**(9): p. 993-9.

31. Nishikawa, R., et al., *Immunohistochemical analysis of the mutant epidermal growth factor, deltaEGFR, in glioblastoma.* Brain tumor pathology, 2004. **21**(2): p. 53-6.

32. Hegi, M.E., et al., *Correlation of O6-methylguanine methyltransferase (MGMT) promoter methylation with clinical outcomes in glioblastoma and clinical strategies to modulate MGMT activity.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2008. **26**(25): p. 4189-99.

33. Nowell, P.C., *The clonal evolution of tumor cell populations.* Science, 1976. **194**(4260): p. 23-8.

34. Greaves, M., *Cancer stem cells: back to Darwin?* Seminars in cancer biology, 2010. **20**(2): p. 65-70.

35. Lyons, J.G., et al., *Clonal diversity in carcinomas: its implications for tumour progression and the contribution made to it by epithelial-mesenchymal transitions.* Clinical & experimental metastasis, 2008. **25**(6): p. 665-77.

36. Gilbertson, R.J. and J.N. Rich, *Making a tumour's bed: glioblastoma stem cells and the vascular niche.* Nature reviews. Cancer, 2007. **7**(10): p. 733-6.

37. Tate, M.C. and M.K. Aghi, *Biology of angiogenesis and invasion in glioma.* Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics, 2009. **6**(3): p. 447-57.

38. Brat, D.J., A.C. Bellail, and E.G. Van Meir, *The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis.* Neuro-oncology, 2005. **7**(2): p. 122-33.

39. Cuddapah, V.A., et al., *A neurocentric perspective on glioma invasion.* Nature reviews. Neuroscience, 2014. **15**(7): p. 455-65.

40. Krakstad, C. and M. Chekenya, *Survival signalling and apoptosis resistance in glioblastomas: opportunities for targeted therapeutics.* Molecular cancer, 2010. **9**: p. 135.

41. Brenner, D. and T.W. Mak, *Mitochondrial cell death effectors.* Current opinion in cell biology, 2009. **21**(6): p. 871-7.

42. Engelman, J.A., *Targeting PI3K signalling in cancer: opportunities, challenges and limitations.* Nature reviews. Cancer, 2009. **9**(8): p. 550-62.

43.   *Comprehensive genomic characterization defines human glioblastoma genes and core pathways.* Nature, 2008. **455**(7216): p. 1061-8.

44.   Siegel, R., D. Naishadham, and A. Jemal, *Cancer statistics, 2013.* CA: a cancer journal for clinicians, 2013. **63**(1): p. 11-30.

45.   Winter, J.M., et al., *Survival after resection of pancreatic adenocarcinoma: results from a single institution over three decades.* Annals of surgical oncology, 2012. **19**(1): p. 169-75.

46.   Ryan, D.P., T.S. Hong, and N. Bardeesy, *Pancreatic adenocarcinoma.* The New England journal of medicine, 2014. **371**(11): p. 1039-49.

47.   Klimstra, D.S., M.B. Pitman, and R.H. Hruban, *An algorithmic approach to the diagnosis of pancreatic neoplasms.* Archives of pathology & laboratory medicine, 2009. **133**(3): p. 454-64.

48.   Klein, A.P., *Genetic susceptibility to pancreatic cancer.* Molecular carcinogenesis, 2012. **51**(1): p. 14-24.

49.   Hruban, R.H., R.E. Wilentz, and S.E. Kern, *Genetic progression in the pancreatic ducts.* The American journal of pathology, 2000. **156**(6): p. 1821-5.

50.   Andreotti, G. and D.T. Silverman, *Occupational risk factors and pancreatic cancer: a review of recent findings.* Molecular carcinogenesis, 2012. **51**(1): p. 98-108.

51.   Makohon-Moore, A. and C.A. Iacobuzio-Donahue, *Pancreatic cancer biology and genetics from an evolutionary perspective.* Nature reviews. Cancer, 2016. **16**(9): p. 553-65.

52.   Jones, S., et al., *Core signaling pathways in human pancreatic cancers revealed by global genomic analyses.* Science, 2008. **321**(5897): p. 1801-6.

53.   Bardeesy, N. and R.A. DePinho, *Pancreatic cancer biology and genetics.* Nature reviews. Cancer, 2002. **2**(12): p. 897-909.

54.   Sakorafas, G.H., A.G. Tsiotou, and G.G. Tsiotos, *Molecular biology of pancreatic cancer; oncogenes, tumour suppressor genes, growth factors, and their receptors from a clinical perspective.* Cancer treatment reviews, 2000. **26**(1): p. 29-52.

55.   Li, D., et al., *Pancreatic cancer.* Lancet, 2004. **363**(9414): p. 1049-57.

56.   Goggins, M., *Molecular markers of early pancreatic cancer.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2005. **23**(20): p. 4524-31.

57.   Goldstein, A.M., et al., *Increased risk of pancreatic cancer in melanoma-prone kindreds with p16INK4 mutations.* The New England journal of medicine, 1995. **333**(15): p. 970-4.

58.   Ziske, C., et al., *Prognostic value of CA 19-9 levels in patients with inoperable adenocarcinoma of the pancreas treated with gemcitabine.* British journal of cancer, 2003. **89**(8): p. 1413-7.

59.   Ghaneh, P., E. Costello, and J.P. Neoptolemos, *Biology and management of pancreatic cancer.* Gut, 2007. **56**(8): p. 1134-52.

60.   Vigneron, N., et al., *Identifying source proteins for MHC class I-presented peptides.* Methods in molecular biology, 2013. **960**: p. 187-207.

61.   Chen, Y.T., A.O. Gure, and M.J. Scanlan, *Serological analysis of expression cDNA libraries (SEREX): an immunoscreening technique for identifying immunogenic tumor antigens.* Methods in molecular medicine, 2005. **103**: p. 207-16.

62.   Schirle, M., et al., *Identification of tumor-associated MHC class I ligands by a novel T cell-independent approach.* European journal of immunology, 2000. **30**(8): p. 2216-25.

63.   Lu, Y.C., et al., *Efficient identification of mutated cancer antigens recognized by T cells associated with durable tumor regressions.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2014. **20**(13): p. 3401-10.

64.   Sarid, R. and S.J. Gao, *Viruses and human cancer: from detection to causality.* Cancer letters, 2011. **305**(2): p. 218-27.

65.   Kenter, G.G., et al., *Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia.* The New England journal of medicine, 2009. **361**(19): p. 1838-47.

66.   Vigneron, N., *Human Tumor Antigens and Cancer Immunotherapy.* BioMed research international, 2015. **2015**: p. 948501.

67.   Balkwill, F.R., M. Capasso, and T. Hagemann, *The tumor microenvironment at a glance.* Journal of cell science, 2012. **125**(Pt 23): p. 5591-6.

68.   Carmeliet, P. and R.K. Jain, *Molecular mechanisms and clinical applications of angiogenesis.* Nature, 2011. **473**(7347): p. 298-307.

69.   Jain, R.K., *Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy.* Science, 2005. **307**(5706): p. 58-62.

70.   Lu, P., et al., *Extracellular matrix degradation and remodeling in development and disease.* Cold Spring Harbor perspectives in biology, 2011. **3**(12).

71.   Fridman, W.H., et al., *The immune contexture in human tumours: impact on clinical outcome.* Nature reviews. Cancer, 2012. **12**(4): p. 298-306.

72.   Hannani, D., et al., *Harnessing gammadelta T cells in anticancer immunotherapy.* Trends in immunology, 2012. **33**(5): p. 199-206.

73.   Andreu, P., et al., *FcRgamma activation regulates inflammation-associated squamous carcinogenesis.* Cancer cell, 2010. **17**(2): p. 121-34.

74.   Lin, E.Y., et al., *Macrophages regulate the angiogenic switch in a mouse model of breast cancer.* Cancer research, 2006. **66**(23): p. 11238-46.

75.   Huang, B., et al., *Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host.* Cancer research, 2006. **66**(2): p. 1123-31.

76.   Paunescu, V., et al., *Tumour-associated fibroblasts and mesenchymal stem cells: more similarities than differences.* Journal of cellular and molecular medicine, 2011. **15**(3): p. 635-46.

77.   Erez, N., et al., *Cancer-Associated Fibroblasts Are Activated in Incipient Neoplasia to Orchestrate Tumor-Promoting Inflammation in an NF-kappaB-Dependent Manner.* Cancer cell, 2010. **17**(2): p. 135-47.

78.   Hicklin, D.J., F.M. Marincola, and S. Ferrone, *HLA class I antigen downregulation in human cancers: T-cell immunotherapy revives an old story.* Molecular medicine today, 1999. **5**(4): p. 178-86.

79.   Johnsen, A.K., et al., *Deficiency of transporter for antigen presentation (TAP) in tumor cells allows evasion of immune surveillance and increases tumorigenesis.* Journal of immunology, 1999. **163**(8): p. 4224-31.

80.   Tsujimoto, Y., et al., *Involvement of the bcl-2 gene in human follicular lymphoma.* Science, 1985. **228**(4706): p. 1440-3.

81.   Minn, A.J., et al., *Expression of bcl-xL can confer a multidrug resistance phenotype.* Blood, 1995. **86**(5): p. 1903-10.

82.   Sakaguchi, S., *Regulatory T cells: key controllers of immunologic self-tolerance.* Cell, 2000. **101**(5): p. 455-8.

83.   Maeda, H. and A. Shiraishi, *TGF-beta contributes to the shift toward Th2-type responses through direct and IL-10-mediated pathways in tumor-bearing mice.* Journal of immunology, 1996. **156**(1): p. 73-8.

84.   Zhou, Q., et al., *Expression of macrophage migration inhibitory factor by neuroblastoma leads to the inhibition of antitumor T cell reactivity in vivo.* Journal of immunology, 2008. **181**(3): p. 1877-86.

85. Nakashima, M., K. Sonoda, and T. Watanabe, *Inhibition of cell growth and induction of apoptotic cell death by the human tumor-associated antigen RCAS1.* Nature medicine, 1999. **5**(8): p. 938-42.

86. Ampie, L., E.C. Woolf, and C. Dardis, *Immunotherapeutic advancements for glioblastoma.* Frontiers in oncology, 2015. **5**: p. 12.

87. Louveau, A., et al., *Structural and functional features of central nervous system lymphatic vessels.* Nature, 2015. **523**(7560): p. 337-41.

88. Coniglio, S.J. and J.E. Segall, *Review: molecular mechanism of microglia stimulated glioblastoma invasion.* Matrix biology : journal of the International Society for Matrix Biology, 2013. **32**(7-8): p. 372-80.

89. Hao, C., et al., *Cytokine and cytokine receptor mRNA expression in human glioblastomas: evidence of Th1, Th2 and Th3 cytokine dysregulation.* Acta neuropathologica, 2002. **103**(2): p. 171-8.

90. Jackson, C., et al., *Challenges in immunotherapy presented by the glioblastoma multiforme microenvironment.* Clinical & developmental immunology, 2011. **2011**: p. 732413.

91. Mittal, S.K. and P.A. Roche, *Suppression of antigen presentation by IL-10.* Current opinion in immunology, 2015. **34**: p. 22-7.

92. Kirkbride, K.C. and G.C. Blobe, *Inhibiting the TGF-beta signalling pathway as a means of cancer immunotherapy.* Expert opinion on biological therapy, 2003. **3**(2): p. 251-61.

93. Sheng, K.C., M.D. Wright, and V. Apostolopoulos, *Inflammatory mediators hold the key to dendritic cell suppression and tumor progression.* Current medicinal chemistry, 2011. **18**(36): p. 5507-18.

94. Dix, A.R., et al., *Immune defects observed in patients with primary malignant brain tumors.* Journal of neuroimmunology, 1999. **100**(1-2): p. 216-32.

95. Bloch, O., et al., *Gliomas promote immunosuppression through induction of B7-H1 expression in tumor-associated macrophages.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2013. **19**(12): p. 3165-75.

96. Jordan, J.T., et al., *Preferential migration of regulatory T cells mediated by glioma-secreted chemokines can be blocked with chemotherapy.* Cancer immunology, immunotherapy : CII, 2008. **57**(1): p. 123-31.

97. Crane, C.A., et al., *Soluble factors secreted by glioblastoma cell lines facilitate recruitment, survival, and expansion of regulatory T cells: implications for immunotherapy.* Neuro-oncology, 2012. **14**(5): p. 584-95.

98. Nomi, T., et al., *Clinical significance and therapeutic potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2007. **13**(7): p. 2151-7.

99. Loos, M., et al., *Expression of the costimulatory molecule B7-H3 is associated with prolonged survival in human pancreatic cancer.* BMC cancer, 2009. **9**: p. 463.

100. Yamato, I., et al., *Clinical importance of B7-H3 expression in human pancreatic cancer.* British journal of cancer, 2009. **101**(10): p. 1709-16.

101. Stupp, R., et al., *Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma.* The New England journal of medicine, 2005. **352**(10): p. 987-96.

102. Jacinto, F.V. and M. Esteller, *MGMT hypermethylation: a prognostic foe, a predictive friend.* DNA repair, 2007. **6**(8): p. 1155-60.

103. Lee, F.Y., et al., *Clinical pharmacokinetics of oral CCNU (lomustine).* Cancer chemotherapy and pharmacology, 1985. **14**(2): p. 125-31.

104. Oettle, H., et al., *Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial.* JAMA, 2007. **297**(3): p. 267-77.

105. Burris, H.A., 3rd, et al., *Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 1997. **15**(6): p. 2403-13.

106. Teague, A., K.H. Lim, and A. Wang-Gillam, *Advanced pancreatic adenocarcinoma: a review of current treatment strategies and developing therapies.* Therapeutic advances in medical oncology, 2015. **7**(2): p. 68-84.

107. Friedman, H.S., et al., *Bevacizumab alone and in combination with irinotecan in recurrent glioblastoma.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2009. **27**(28): p. 4733-40.

108. Nagane, M., et al., *A common mutant epidermal growth factor receptor confers enhanced tumorigenicity on human glioblastoma cells by increasing proliferation and reducing apoptosis.* Cancer research, 1996. **56**(21): p. 5079-86.

109. Hasselbalch, B., et al., *Cetuximab, bevacizumab, and irinotecan for patients with primary glioblastoma and progression after radiation therapy and temozolomide: a phase II trial.* Neuro-oncology, 2010. **12**(5): p. 508-16.

110. Moore, M.J., et al., *Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2007. **25**(15): p. 1960-6.

111. Rampling, R., et al., *A Cancer Research UK First Time in Human Phase I Trial of IMA950 (Novel Multipeptide Therapeutic Vaccine) in Patients with Newly Diagnosed Glioblastoma.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2016. **22**(19): p. 4776-4785.

112. Phuphanich, S., et al., *Phase I trial of a multi-epitope-pulsed dendritic cell vaccine for patients with newly diagnosed glioblastoma.* Cancer immunology, immunotherapy : CII, 2013. **62**(1): p. 125-35.

113. Gjertsen, M.K., et al., *Vaccination with mutant ras peptides and induction of T-cell responsiveness in pancreatic carcinoma patients carrying the corresponding RAS mutation.* Lancet, 1995. **346**(8987): p. 1399-400.

114. Crane, C.A., et al., *Individual patient-specific immunity against high-grade glioma after vaccination with autologous tumor derived peptides bound to the 96 KD chaperone protein.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2013. **19**(1): p. 205-14.

115. Prins, R.M., T.F. Cloughesy, and L.M. Liau, *Cytomegalovirus immunity after vaccination with autologous glioblastoma lysate.* The New England journal of medicine, 2008. **359**(5): p. 539-41.

116. Schirrmacher, V., et al., *Human tumor cell modification by virus infection: an efficient and safe way to produce cancer vaccine with pleiotropic immune stimulatory properties when using Newcastle disease virus.* Gene therapy, 1999. **6**(1): p. 63-73.

117. Laheru, D., et al., *Allogeneic granulocyte macrophage colony-stimulating factor-secreting tumor immunotherapy alone or in sequence with cyclophosphamide for metastatic pancreatic cancer: a pilot study of safety, feasibility, and immune activation.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2008. **14**(5): p. 1455-63.

118. Hodi, F.S., et al., *Improved survival with ipilimumab in patients with metastatic melanoma.* The New England journal of medicine, 2010. **363**(8): p. 711-23.

119. Topalian, S.L., et al., *Safety, activity, and immune correlates of anti-PD-1 antibody in cancer.* The New England journal of medicine, 2012. **366**(26): p. 2443-54.

120. Kolb, H.J., et al., *Donor leukocyte transfusions for treatment of recurrent chronic myelogenous leukemia in marrow transplant patients.* Blood, 1990. **76**(12): p. 2462-5.

121. Dudley, M.E., et al., *Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes.* Science, 2002. **298**(5594): p. 850-4.

122. Morgan, R.A., et al., *Cancer regression in patients after transfer of genetically engineered lymphocytes.* Science, 2006. **314**(5796): p. 126-9.

123. Gilboa, E., *How tumors escape immune destruction and what we can do about it.* Cancer immunology, immunotherapy : CII, 1999. **48**(7): p. 382-5.

124. Lee, P.P., et al., *Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients.* Nature medicine, 1999. **5**(6): p. 677-85.

125. Romero, P., et al., *Ex vivo staining of metastatic lymph nodes by class I major histocompatibility complex tetramers reveals high numbers of antigen-experienced tumor-specific cytolytic T lymphocytes.* The Journal of experimental medicine, 1998. **188**(9): p. 1641-50.

126. Quattrocchi, K.B., et al., *Pilot study of local autologous tumor infiltrating lymphocytes for the treatment of recurrent malignant gliomas.* Journal of neuro-oncology, 1999. **45**(2): p. 141-57.

127. Rosenberg, S.A., et al., *Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2011. **17**(13): p. 4550-7.

128. Imataki, O., et al., *IL-21 can supplement suboptimal Lck-independent MAPK activation in a STAT-3-dependent manner in human CD8(+) T cells.* Journal of immunology, 2012. **188**(4): p. 1609-19.

129. Butler, M.O., et al., *Long-lived antitumor CD8+ lymphocytes for adoptive therapy generated using an artificial antigen-presenting cell.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2007. **13**(6): p. 1857-67.

130. Rosenberg, S.A., *Overcoming obstacles to the effective immunotherapy of human cancer.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(35): p. 12643-4.

131. Berger, C., et al., *Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates.* The Journal of clinical investigation, 2008. **118**(1): p. 294-305.

132. Wherry, E.J., et al., *Lineage relationship and protective immunity of memory CD8 T cell subsets.* Nature immunology, 2003. **4**(3): p. 225-34.

133. Chapuis, A.G., et al., *Transferred melanoma-specific CD8+ T cells persist, mediate tumor regression, and acquire central memory phenotype.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(12): p. 4592-7.

134. Sahin, U., et al., *Expression of cancer testis genes in human brain tumors.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2000. **6**(10): p. 3916-22.

135. Corso, C.D., A.N. Ali, and R. Diaz, *Radiation-induced tumor neoantigens: imaging and therapeutic implications.* American journal of cancer research, 2011. **1**(3): p. 390-412.

136. Fujiwara, S., et al., *NY-ESO-1 antibody as a novel tumour marker of gastric cancer.* British journal of cancer, 2013. **108**(5): p. 1119-25.

137. Uemura, N., et al., *Correlation between anti-survivin antibody and survivin mRNA expression in head and neck cancer patients.* Acta oto-laryngologica, 2010. **130**(8): p. 959-65.

138. Robbins, P.F., et al., *A pilot trial using lymphocytes genetically engineered with an NY-ESO-1-reactive T-cell receptor: long-term follow-up and correlates with*

response. Clinical cancer research : an official journal of the American Association for Cancer Research, 2015. **21**(5): p. 1019-27.

139. Everson, R.G., et al., *Efficacy of systemic adoptive transfer immunotherapy targeting NY-ESO-1 for glioblastoma.* Neuro-oncology, 2016. **18**(3): p. 368-78.

140. Gupta, A., et al., *A novel human-derived antibody against NY-ESO-1 improves the efficacy of chemotherapy.* Cancer immunity, 2013. **13**: p. 3.

141. Dutoit, V., et al., *Multiepitope CD8(+) T cell response to a NY-ESO-1 peptide vaccine results in imprecise tumor targeting.* The Journal of clinical investigation, 2002. **110**(12): p. 1813-22.

142. Tsurushima, H., et al., *Reduction of end-stage malignant glioma by injection with autologous cytotoxic T lymphocytes.* Japanese journal of cancer research : Gann, 1999. **90**(5): p. 536-45.

143. Rosenberg, S.A., et al., *Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer.* The New England journal of medicine, 1985. **313**(23): p. 1485-92.

144. Hayes, R.L., et al., *Improved long term survival after intracavitary interleukin-2 and lymphokine-activated killer cells for adults with recurrent malignant glioma.* Cancer, 1995. **76**(5): p. 840-52.

145. Dillman, R.O., et al., *Intralesional lymphokine-activated killer cells as adjuvant therapy for primary glioblastoma.* Journal of immunotherapy, 2009. **32**(9): p. 914-9.

146. Kitahara, T., et al., *Establishment of interleukin 2 dependent cytotoxic T lymphocyte cell line specific for autologous brain tumor and its intracranial administration for therapy of the tumor.* Journal of neuro-oncology, 1987. **4**(4): p. 329-36.

147. Kanwar, R.K., et al., *Recent advances in anti-survivin treatments for cancer.* Current medicinal chemistry, 2010. **17**(15): p. 1509-15.

148. Johnson, M.D., F. Vito, and M.J. O'Connell, *Mesothelin expression in the leptomeninges and meningiomas.* The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society, 2008. **56**(6): p. 579-85.

149. Tozbikian, G., et al., *Mesothelin expression in triple negative breast carcinomas correlates significantly with basal-like phenotype, distant metastases and decreased survival.* PloS one, 2014. **9**(12): p. e114900.

150. Kachala, S.S., et al., *Mesothelin overexpression is a marker of tumor aggressiveness and is associated with reduced recurrence-free and overall survival in early-stage lung adenocarcinoma.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2014. **20**(4): p. 1020-8.

151. Thomas, A., et al., *High mesothelin expression in advanced lung adenocarcinoma is associated with KRAS mutations and a poor prognosis.* Oncotarget, 2015. **6**(13): p. 11694-703.

152. Renaud, S., et al., *Prognostic value of the KRAS G12V mutation in 841 surgically resected Caucasian lung adenocarcinoma cases.* British journal of cancer, 2015. **113**(8): p. 1206-15.

153. Rizk, N.P., et al., *Tissue and serum mesothelin are potential markers of neoplastic progression in Barrett's associated esophageal adenocarcinoma.* Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 2012. **21**(3): p. 482-6.

154. Iwahori, K., et al., *Megakaryocyte potentiating factor as a tumor marker of malignant pleural mesothelioma: evaluation in comparison with mesothelin.* Lung cancer, 2008. **62**(1): p. 45-54.

155. Bafna, S., S. Kaur, and S.K. Batra, *Membrane-bound mucins: the mechanistic basis for alterations in the growth and survival of cancer cells.* Oncogene, 2010. **29**(20): p. 2893-904.

156. Illei, P.B., et al., *Mesothelin Expression in Advanced Gastroesophageal Cancer Represents a Novel Target for Immunotherapy.* Applied immunohistochemistry & molecular morphology : AIMM, 2016. **24**(4): p. 246-52.

157. Hassan, R., et al., *Phase II clinical trial of amatuximab, a chimeric antimesothelin antibody with pemetrexed and cisplatin in advanced unresectable pleural mesothelioma.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2014. **20**(23): p. 5927-36.

158. Fujisaka, Y., et al., *Phase I study of amatuximab, a novel monoclonal antibody to mesothelin, in Japanese patients with advanced solid tumors.* Investigational new drugs, 2015. **33**(2): p. 380-8.

159. Lutz, E., et al., *A lethally irradiated allogeneic granulocyte-macrophage colony stimulating factor-secreting tumor vaccine for pancreatic adenocarcinoma. A Phase II trial of safety, efficacy, and immune activation.* Annals of surgery, 2011. **253**(2): p. 328-35.

160. Bellone, M. and A. Calcinotto, *Ways to enhance lymphocyte trafficking into tumors and fitness of tumor infiltrating lymphocytes.* Frontiers in oncology, 2013. **3**: p. 231.

161. McGuire, S., *World Cancer Report 2014. Geneva, Switzerland: World Health Organization, International Agency for Research on Cancer, WHO Press, 2015.* Advances in nutrition, 2016. **7**(2): p. 418-9.