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Fibroblast activation protein and the local immunosuppression in glioblastoma
Fibroblastový aktivační protein a lokální imunosuprese v glioblastomu

Diploma thesis

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Prohlášení:

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Podpis

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List of abbrevations

Acc - accutase

APCs - antigen presenting cells

ATRX - ATP-dependent helicase

BBB - blood brain barrier

BMDM - Bone marrow derived macrophages

CAFs - Cancer associated fibroblasts

CCL2 - Chemokine C-C motif ligand 2

CD - clusters of diferenciation

CNS - the central nervous system

CSCs - Cancer stem cells

CTLA4 - Cytotoxic T-lymphocyte-associated protein 4

DNA - deoxyribonucleic acid

DMEM - Dulbecco's modified eagle's medium

ECM - extracellular matrix

EDTA - ethylene diamine tetraacetic acid

EGF - Epidermal growth factor

EGFR - Epidermal growth factor receptor

FAP - Fibroblast activation Protein α

Fas - Fas receptor

FasL - Fas ligand

FMO - Fluorescence minus one control

FoxP3 - Forkhead box P3

FBS - fetal bovine serum

FSC - Forward scatter parameter

GAMs - glioma associated myeolid populations

GBM IDH-M - glioblastoma with isocitrate dehydrogenase mutated

GBM IDH-WT - glioblastoma with isocitrate dehydrogenase wild-type

GITR - Glucocorticoid-induced TNFR-related protein

GM-CSF - Granulocyte-macrophage colony stimulating factor

IDH - isocitrate dehydrogenase

IL - Interleukin

KW - Kruskal-Wallis test

LLT1 - Lectin like transcript 1

MHC - Major histocompatibility complex

MV - mean value

M-CSF - Macrophage colony stimulating factor

NCAM1 - Neural Adhesion Molecule 1

NK - Natural killer cells

NKG2D - Natural killer group 2D

NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B

PBS - phosphate buffered saline

PD-1 - Programmed death 1

PD-L1 - Programmed death-ligand 1

PTEN - Phosphatase and tensin homolog

RPMI - Roswell Park Memorial Institute 1640 Medium

SD - standart deviation

SSC - Side Scatter parameter

STAT3 - Signal transducer and activator of transcription 3

STI-1 - Stress-inducible protein 1

TGF-β -Transforming growth factor β

p53 - protein p53

TTD - Tumor tissue disocation kit

Tregs - Regulatory T-cells

VEGF - Vascular endothelial growth factor

WHO - the World Health Organization

WT - wild type

Abstract

Glioblastoma multiforme (GBM) is the most aggressive type of primary brain tumor. Current treatment includes surgical resection with following radio/chemotherapy, but prognosis of patients remains poor with median survival only about 15 months. GBM is characteristic for necrotic regions, abnormal vascularization and strong immunosuppression. Dynamic interactions of cancer cells, immune cells and other stromal cells in the tumor microenvironment can promote tumor growth and progression.

Fibroblast activation protein α (FAP) is overexpressed by the cells in tumor tissue. FAP is important in angiogenesis, remodelation of extracellular matrix and immunomodulation in cancers. The role of FAP in the tumor microenvironment is the subject of recent research.

The aim of the thesis was to prepare a syngeneic mouse model of glioblastoma with and without FAP expression, implement and optimalize the dissociation method for GBM tumor tissue and detect a variety of infiltrated immune cell populations in the GBM microenvironment by flow cytometry.

Optimization of dissociation protocol for glioblastoma tissue was a crucial step for viable cell suspension required for cytometry study of immune cell populations. A combination of dissection by dissociator and enzymatic digestion with mild enzymes was found to be the most suitable method. Syngeneic mouse models of GBM C57BL/6J WT and C57BL/6J FAP-/- implanted intracranially with mouse glioma cells were successfully established and infiltrated immune cell populations were explored. This thesis is pilot study of FAP expression and GBM immunosuppression association. Better understanding of the role of FAP in tumor microenvironment is necessary for development of novel treatment strategies for GBM.

Abstrakt

Multiformní glioblastom (GBM) je nejagresívnějším primárním mozkovým nádorem. Současná terapie je založena především na radikální resekci nádoru a následné chemo/radioterapii, přesto prognóza pacientů zůstává velmi nepříznivá s mediánem prežití pouze 15 měsíců. GBM se vyznačuje výskytem nekróz, abnormální vaskularizací a imunosupresií. Dynamické interakce nádorových buněk, imunitných buněk, ale i ostatných stromálních buněk v nádorovém mikroprostředí mohou vést k nádorovému růstu a progresi.

Fibroblastový aktivačný protein α (FAP) je zvýšeně exprimován v nádorových tkáních. FAP hraje významnou roli v angiogenezi, remodelaci extracelulární matrix a imunomodulaci v nádorové tkáni. Úloha FAP v nádorovém mikroprostředí je předmětem současného výzkumu.

Cílem diplomové práce bylo připravit glioblastomový syngenní myší model s/bez exprese FAP, zavést a optimalizovat disociační metody GBM tkáně a detekovat v ní jednotlivé populace imunitních buněk pomocí průtokové cytometrie.

Optimalizace disociačního protokolu pro tkáň glioblastomu byla kruciálním krokem v přípravě viabilní buněčné suspenze vhodné pro cytometrickou analýzu imunitních buněk. Buněčné suspenze s nejvyšší viabilitou bylo dosaženo pomocí disociace GBM tkáně za použití kombinace disociátoru a enzymů s mírnější enzymovou aktivitou. S úspěchem byly vyvinuty myší syngenní ortotopické GBM modely C57BL/6J WT a C57BL/6J FAP-/- u nichž byly detekovány jednotlivé populace imunitních buněk. Předkládaná diplomová práce je pilotní studií asociace exprese FAP a imunosuprese v modelu GBM. Lepší porozumění role FAP v nádorovém mikroprostředí by mohlo přispět k nalezení nových strategií v léčbě GBM.

Table of Contents

1. Glioblastoma Multiforme

Glioblastoma multiforme (GBM) is one of the most common malignant tumors in the central nervous system (CNS). It represents one of the deadliest tumors with therapeutic resistance and high tumor reccurence. The current therapy includes surgical resection and radio/chemotherapy but the patient prognosis still remains poor with the median survival only about 15 months from diagnosis (reviewed in Louis et al., 2016). Global incidence of GBM is less than 2 - 3 per 100,000 people (reviewed in GBD 2016 Brain and Other CNS Cancer Collaborators et al., 2019). Incidence peak is between 55 to 66 years. GBM is more common in men than women (Ohgaki & Kleihues, 2005). The etiology is still unknown, genetic predisposition was observed only in 10 % of patients but the it is probably a result of mutation in more genes associated with proteins of DNA repair (reviewed in Fisher, Schwartzbaum, Wrensch, & Wiemels, 2007; Kyritsis, Bondy, Rao, & Sioka, 2010).

The typical feature of GBM is cellular tumor heterogeneity characterized by genetic alterations with an ability to dedifferentiate transformed cells into a stem cell-like state (P. Wang, Wan, Xiong, Feng, & Wu, 2017). GBM is developed from astroglia and are classified as a Grade 4 astrocytoma according to The World Health Organization (WHO) (reviewed in Louis et al., 2016).

There are two types of GBM divided according to mutation in isocitrate dehydrogenase (IDH): tumor developed de novo from progenitor cells defined as primary GBM with wild-type IDH (GBM IDH-WT) and tumor developed from preexisting lower grade astrocytoma tumor defined as secondary GBM with mutated IDH (GBM IDH-M). Typical genetic features of GBM IDH-WT are upregulation of Vascular Endothelial Growth factor (VEGF), Epidermal Growth factor receptor (EGFR)and mutation in Phosphatase and Tensin Homolog (PTEN) (Godard et al., 2003; Lai et al., 2011; Ohgaki & Kleihues, 2007). GBM IDH-M lacks epidermal growth factor amplifications and mutations in protein p53 and ATP-dependent helicase (ATRX) (Liu et al., 2012; Nobusawa, Watanabe, Kleihues, & Ohgaki, 2009). Both types of GBM are typical for loss of heterozygosity 10q (Ohgaki et al., 2004). GBM tumors may have various expression profiles and can be divided into neural, pro-neural, classical and

mesenchymal subgroup. Response to current therapy in patients differs with particular subtype of GBM (Verhaak et al., 2010).

2. Glioblastoma microenvironment

Hypoxic microenvironment with abnormal vascularization is typical for GBM. GBM microenvironment consists of malignant and non-malignant stromal cells which interact through cell contacts and soluble factors. Secreted mediators and cell interactions among cells in GBM microenvironment may promote growth and progression of tumor.

Hypoxia is one of the main hallmarks of solid tumors (Bhandari et al., 2019). Hypoxic microenvironment induces immune evasion and angiogenesis of disorganized blood vessels with abnormal morphology in the tumor site a necessity of increasing oxygen utilization (M. Z. Noman et al., 2011; Muhammad Zaeem Noman et al., 2009). GBM is one of the most vascularized tumors with upregulation of proangiogenic factors. Overproduction of Vascular endothelial growth factor (VEGF) leads to impaired function of endothelial cells and pericytes. Abnormal formation of blood vessels is associated with further hypoxic environment, necrosis and disruption of the blood brain barrier (BBB) integrity (Leten, Struys, Dresselaers, & Himmelreich, 2014).

Acidic surroundings in the tumor increase genomic instability and accumulation of gene mutations in Cancer Stem Cells (CSCs) by downregulation of DNA repair genes (Bindra et al., 2005; Bindra & Glazer, 2007; BINDRA et al., 2005). CSCs are maintained in undifferentiated stem cell state with self-renewal activity by hypoxic tumor microenvironment (P. Li, Zhou, Xu, & Xiao, 2013). They promote initiation, maintenance, progression and reccurence of the tumor (reviewed in Ayob & Ramasamy, 2018).

Further progression of the tumor is mediated through interaction of transformed cells, immune subpopulations and other populations of stromal cells with associated remodelation and degradation of extracellular matrix (ECM).

Secretion of chemotactic molecules in the tumor microenvironment induce attraction of infiltrated immune cells like Microglia, Bone Marrow Derived Macrophages (BMDM), T Regulatory cells (Treg), conventional T cells and Natural

killer cells (NK). Hypoxia further triggers Signal Transducer and Activator of Transcription 3 (STAT3) expression in immune cells and induces anti-inflammatory response (Wei et al., 2011). Dynamic interactions among immune and tumor cells induce immunosuppressive properties of tumor microenvironment. Immunosuppressive mechanisms are mediated through membrane molecules (Cytotoxic T-lymphocyte-Associated Protein 4 (CTLA4), Fas ligand (FasL)) or soluble factors (Interleukin 10 (IL-10), Transforming Growth Factor β (TGF-β), Chemokine C-C motif ligand 2 (CCL2) and other chemotactic molecules) to induce apoptosis of T effector cells and promote immunosuppressive phenotype in immune cells. Immunosuppression in the GBM microenvironment promotes tumor growth and progression.

Remodelation and degradation of ECM induce activation of stromal cells in the tumor microenvironment. One of the markers of activated stroma and ECM remodelation is a protease Fibroblast activation protein α (FAP). In the tumor microenvironment, FAP is mainly expressed by mesenchymal cells, cancer cells and small subsets of immune cells (Busek et al., 2016). Expression of FAP is elevated in GBM and increases invasiveness of the tumor (Mentlein, Hattermann, Hemion, Jungbluth, & Held-Feindt, 2011).

2.1. Fibroblast activation protein α

Proteases in the context of tumor microenvironment, have become a hotspot of recent studies. Variety of tumors, including GBM, express high levels of Fibroblast activation protein α (FAP), especially in mesenchymal type of GBM (Busek et al., 2016; F. Liu et al., 2015). It may be present in transmembrane intra/extracellulary as a soluble form in plasma. FAP expression in physiological conditions in adult tissue is very low or undetectable but it is overexpressed in tumor tissue (F. Liu et al., 2015). High levels of FAP correlate with poor outome of tumor diseases (López et al., 2016; Wikberg et al., 2013).

FAP is a serine protease belonging to the dipeptidyl peptidase family with gelatinase endopeptidase and post-proline expopeptidase activity (Goldstein et al., 1997; Rettig et al., 1988; Scanlan et al., 1994). FAP is a homodimer consisting of transmembrane domain with intracellular cytoplasmic tail and extracellular domain with active site (Scanlan et al., 1994). Soluble active form of FAP is detected in plasma and

is able to cleave antiplasmin but its function in tumors is still unknown (Lee et al., 2006). FAP is expressed during ontogeny, otherwise protein is rarely detected in healthy adult tissues with the exception of stromal cells in bone marrow, pancreatic alpha cells and cells in uterine stroma (Bae et al., 2008; Busek, Hrabal, Fric, & Sedo, 2015; Dolznig et al., 2005; Niedermeyer et al., 2001). Nevertheless, FAP is overexpressed at site of tissue remodeling in wound healing and cancer (Jacob, Chang, & Puré, 2012; Kelly, Huang, Simms, & Mazur, 2012). In the tumor microenvironment, FAP is mainly expressed by Cancer associated fibroblasts (CAFs) (Huang et al., 2017). It was shown that CAFs promote tumor progression by realeasing growth and angiogenic factors, maintain immunosuppression and epithelial-mesenchymal transition (Al-Ansari et al., 2012; Brentnall, 2012; Labernadie et al., 2017; Orimo et al., 2005; Ziani, Chouaib, & Thiery, 2018). FAP is upregulated in astroglial tumors (Mentlein et al., 2011). Transmemembrane FAP expression in tumor site or soluble form in plasma correspond with poor prognosis and worse patient survival (F. Liu et al., 2015). In the GBM microenvironment, FAP was detected on mesenchymal cells (endothelial cells), astrocytes, neural stem cells, subpopulation of CD45+ cells but mainly on cancer cells (Busek et al., 2016). Some subsets of macrophages may have expressed FAP as it was described in lung and human breast cancer (Arnold, Magiera, Kraman, & Fearon, 2014; Tchou et al., 2013).

2.2. The Immunosuppression in Glioblastoma

The GBM microenvironment is typical for immunosuppression maintained by dynamic cell interactions of immune cells, soluable factors but also by cancer cells. Immunosuppression of tumor microenvironment is maintained mainly by myeloid cell populations (microglia) and infiltrated (BMDM) and lymphocytes (mainly Treg and NK cells). Due to the conditions of the tumor microenvironment, the immune response is shifted to Th2 tumor supportive response leading to further tumor progression (Kumar et al., 2006).

GBM cells promote apoptosis of T cells and immunosuppression by various cell contact interactions. GBM cells express Fas ligand (FasL) interacting with Fas receptor (Fas) expressed on T effector cells (Gratas et al., 1997). Tumor necrosis factor CD70, known to be overexpressed in gliomas, trasmits death signal through interaction of

CD27 on T cells (Wischhusen et al., 2002). The immune checkpoint molecule Program death 1 (PD-1) expressed by GBM cells suppresses T cell activity (Dong et al., 2002). GBM cells overexpress Galectin-1 and suppress antitumor activity of NK cells (G. J. Baker et al., 2014). Furthermore, GBM cells overexpress ligand Lectin-like transcript 1 (LLT1) for inhibitory receptors of NK cells, whereas its expression in the brain under physiological conditions is very low (Roth et al., 2007).

Compared to the other types of brain cancer, GBM is more frequently infiltrated by NK cells (I. Yang, Han, Sughrue, Tihan, & Parsa, 2011). They are mostly present in perivascular and extratumoral site. The inhibitory receptors of NK cells recognize downregulation of major histocompatibility complex I (MHC), often seen in cancer cells. However, GBM cells express MHCI molecules and do not potentiate activation of NK cells (Kmiecik et al., 2013). Cytotoxic response of NK cells is mediated throug expression of FcγRIII (CD16) and Natural killer group 2D (NKG2D) receptors. The GBM microenvironment decreases the expression of NKG2D on NK cells and inhibit their activation by production of TGFβ (Crane et al., 2010; Friese et al., 2004).

Production of soluable factors at tumor site promotes infiltration of immune cells and recruits them for the tumor supportive phenotype.

Treg are attracted to GBM microenvironment by chemokines like chemokine C-C motif ligand 2 (CCL2), mainly produced by tumor cells (A. L. Chang et al., 2016). Immunosuppressive effects of Treg include expression of surface markers and secretion of soluble modulators. Treg produce IL-10 and TGF-β which downregulate the expression of inflammatory cytokines and activation of macrophages, thus suppressing immune response against tumor cells (Jarnicki, Lysaght, Todryk, & Mills, 2006). Production of IL-10 may block Nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) activity needed in inflammatory response during cell stress (Driessler, Venstrom, Sabat, Asadullah, & Schottelius, 2004). One of the direct immunosuppressive mechanisms of Treg is through expression cytotoxic T-lymphocyte-associated protein 4 (CTLA4). The CTLA4 molecule interacts with costimulation molecules on macrophages and prevents activation of T effector cells (Takahashi et al., 2000). Immune response of T effector cells and activated phenotype of macrophages is suppressed as they do not receive second signal, which is important for development of effective immune response (Vandenborre et al., 1999).

Microglia and BMDM acquire anti-inflammatory M2 like phenotype after exposure to glioma derived factors, like IL-10 and TGFβ. M2 microglia and BMDM highly express STAT3 and are recruited to be glioma associated myeolid populations (GAMs) (Yi et al., 2011). They are attracted to lesions of tumor by various chemokines secreted in tumor microenvironment. One of the main chemoattractant increasing GAMs infiltration is CCL2 secreted by tumor cells. Accumulation of GAMs in tumor site may be also promoted by tumor secreted Stroma-derived factor 1 (SDF-1), Macrophage colony-stimulating factor (M-CSF), Granulocyte-macrophage colony-stimulating factor (GM-CSF) and EGF (Sielska et al., 2013; S.-C. Wang, Hong, Hsueh, & Chiang, 2012). GAMs promote invasive properties of tumor cells, support neovascularization and contribute to immunosuppression in the tumor microenvironment. GAMs produce VEGF, IL-6, stress-inducible protein 1 (STI-1), IL-1β, and Epidermal Growth Factor (EGF) to enhance recruitment of endothelial progenitor cells and invasion of glioma cells (Carvalho da Fonseca et al., 2014; Coniglio et al., 2012; Osterberg et al., 2016; L. Zhang et al., 2017). Secretion of anti-inflammatory cytokines decreases expression of MHCII, costimulatory molecules and inhibits activity of antigen presenting cells (APCs) and T cells.

3. Phenotyping of Immune Cells for Flow Cytometry

Flow cytometry is a powerful tool for analysis of large quantities of cells with speed and relative precision. Characterization of a tumor includes detection of various infiltrated cell types in the tumor microenvironment which may play a significant role in tumorogenesis.

3.1. Regulatory T Cells

Regulatory T cells (Treg) are a subpopulation of lymphocytes. They maintain immune response against self and non self antigens by suppression by T effector cell induction and proliferation and prevent development of various autoimmune diseases. (Bettelli et al., 2006). Under physiological conditions, the frequency of Treg in human is about 5 % of all circulating CD4 T cells in peripheral blood and 13 % of spleen-localized Treg in mouse (Rodríguez-Perea, Arcia, Rueda, & Velilla, 2016). There are two populations of Treg, thymus-derived natural Treg (nTreg) and induced Treg (iTreg)

derived from peripheral naive CD4 T cells (Adeegbe & Nishikawa, 2013). The most frequently represented population of Treg are *de novo* derived nTreg (Wainwright, Sengupta, Han, & Lesniak, 2011).

This CD4 T cell subset is characterized by the higher expression of CD25, (receptor for IL2) than other lymphocytes (Baecher-Allan, Brown, Freeman, & Hafler, 2001). By the overexpression of IL2 receptor, Treg obtain IL2 more avidly than other T cells and cause induction of cytokine deprivation-mediated apoptosis of T cells (Pandiyan, Zheng, Ishihara, Reed, & Lenardo, 2007).

Transcription factor forkhead box P3 (FoxP3), master regulator in development and function of Treg, has been considered as a very specific marker. Some authors have suggested that FoxP3 may be expressed by non-hematopoetic cells, like epithelial cells (X. Chang et al., 2005; Zuo et al., 2007). However most of CD4+CD25+ cells express FoxP3 (Roncador et al., 2005). Another transcription factor crucial for functional stability of Treg is Helios (Sebastian et al., 2016). Both FoxP3 and Helios are markers of the activated Treg in human and rodents and enhance their immunosuppressive function (X. Li et al., 2018; Sayour et al., 2015).

Glucocorticoid-induced TNFR-related protein (GITR) molecule is important for thymic development and expansion of Treg, simultaneously expression of GITR may result in loss of FoxP3 expression, thus reduction of Treg population (Ephrem et al., 2013; Mahmud et al., 2014).

Some of the dysfunctional Treg express PD-1 (Lowther et al., 2016). Whereas in healthy humans PD-1+ Treg make up only about 4 % of Treg population, in mice it can be more than 50 % (Rodríguez-Perea et al., 2016). Detecting Treg by PD-1 may be questionable as some authors have reported that most of the Treg in mouse lymphoid tissue internalize PD-1 and lose its surface expression (Raimondi, Shufesky, Tokita, Morelli, & Thomson, 2006).

Identification of Treg by CTLA4, GITR and PD-1 can be controversial, since expression of these markers is not constant and depends on the functional state and tissue location of Treg.

The basic cytometry antibody panel for detection of human and mouse Treg should be constitued at least by CD4, CD25 markers and by intracelullar molecules FoxP3 and Helios.

3.2. Natural Killer Cells

Natural killer cells (NK) are a subpopulation of innate cells originating in lymphoid lineage. NK cells are developed from common lymphoid precursor in primary and secondary lymphoid organs. They lack antigen specific cell surface receptors and provide rapid immune response without previous activation. They are important in immune response against pathogens and neoplastic cells and are the first effector cells at inflammation sites. Through their activating and inibitory receptors, NK cells are able to recognize stressed and cancer cells with impaired MHC molecules expression (reviewed in Vivier et al., 2011).

Neural adhesion molecule 1 (NCAM1), known as CD56, is often used for detection of NK cells (reviewed in Poli et al., 2009). NK cells express activating and inhibitory receptors which are frequently used as markers. Activating receptor CD16 is responsible for cytotoxic response. The most of peripheral NK cells are CD56 and CD16 positive, however, using CD56 as marker for NK cells in brain tumors is problematic because it is expressed in neuronal tissue as well (Amand et al., 2017; Gattenlöhner et al., 2009). Other markers used for NK cells detection are natural cytotoxicity receptors NKp30 (CD337), NKp44 (CD336) and most commonly used NKp46 (CD335) (Walzer et al., 2007). NK cells belong to Group 3 innate lymphoid cells and are negative for CD3 marker.

In both humans and mice, markers frequently used for detection of NK cells in a brain by flow cytometry include CD16 or CD335. The basic panel for flow cytometry should also include CD3 as a negative marker.

3.3. Myeloid Populations

The main immune cell population maintaining homeostasis in the CNS is exclusively brain tissue resident myeolid cells - microglia. Under pathological conditions, microglia are activated and together with infiltrated bone marrow derived

macrophages (BMDM) restore the brain equilibrium (reviewed in Prinz & Priller, 2017).

The frequency of microglia is about 10–15% from all cells found in brain (Lawson, Perry, & Gordon, 1992). Microglia have function in brain development and maintenance of physiological conditions in neural environment. The main role of microglia is phagocytosis of apoptotic bodies from dead cells and invading virus or bacteria. Activated microglia become efficient antigen presenting cells (APCs) (Hickey & Kimura, 1988). Post-inflammation microglia help repair damaged neural circuits and promote regrowth of neural tissue (Gehrmann, Matsumoto, & Kreutzberg, 1995).

Microglia and BMDM represent two ontogenetically separate myeolid populations with different origin but similiar immunomodulating functions. Microglia derive from the progenitors of the yolk sac in early embryogenesis and populate the brain parenchyma while BMDM arise from hematopoietic stem cells (Ginhoux et al., 2010).

General markers for detection of myeloid populations by flow cytometry are CD11b, CD68, Iba1 bith for mice and humans and mouse specific F4/80. Typical markers for pro-inflammatory M1 like myeolid cells are MHCII and costimulatory molecules. Tumor supportive M2 myeloid populations may be detected by expression of CD163, CD204 and CD206. Both microglia and BMDM express CD45 but with different intensity which is used for separation of resident Microglia (CD45low) from BMDM (CD45high). Microglia can be distinguished from another myeloid populations by TMEM119 molecule, which is a specific surface marker for brain resident myeloid cells (Bennett et al., 2016; Satoh et al., 2016).

The antibody panel for basic detection of myeloid population should consist of their general markers CD11b, CD68, Iba1 (for mice and humans) and F4/80 (for mice) which are expressed both by microglia and BMDM.

4. Dissociation Methods for Tumor Tissue

In order to acquire authentical data, it is essential to have the proper single cell suspension for measuring fluorescence of labelled antibodies which accurately target specific markers of cell subtypes. Creating such single cell suspension requires effective

preparation of the tissue. The approaches for processing of tissue to single cell suspension vary and depend on the type and state of the tissue. Dissociation of the tissue to single cell suspension may be done either mechanically or enzymatically.

The main purpose of creating single cell suspension should be preservation of cell viability and cellular diversity while maintaining qualitative and quantitative characteristics of population markers to provide the most reliable cytometry data. Before the dissociation of tissue to single cell suspension requierd for the flow cytometry, the features of the given tissue and detected cell populations need to be considered carefully.

GBM is an invading type of tumor with highly necrotic areas and dissorganized blood vessels. Immune cell populations are lightly adherent cells that should be treated either with mild enzymes for a short period of time, or with no enzymes at all in order to maintain cell viability and preserve expression of the markers.

4.1. Enzymatical Dissociation

Selected enzymes should digest only proteins of ECM, proteins of tight junctions between cells, and DNA fragments released from dead cells without altering the expression of surface markers.

Papain

Papain is a cystein peptidase C1 protease that mainly digests proteins of tight junctions between cells. Papain was used for isolation of human microglia, astrocytes for cell culture and mouse tumor cells of medulloblastoma (Oliver et al., 2005; Rustenhoven et al., 2016; Y. Zhang et al., 2016). However, digestion by papain leads to contamination of cell suspension by DNA fragments released from lysed cells during enzymatic breakdown, which results in the loss of viable cells (Pistollato et al., 2007; Stremnitzer et al., 2015; Veshchev et al., 2016).

Deoxyribonuclease

Deoxyribonuclease (DNAse) is another enzyme that should be included in the mixture of enzymes as it prevents clumping of the cells by degradation of released DNA fragment from dead cells (Price, 1975).

Collagenase

Collagenase is effective for degradation of peptide bonds in collagen, the protein of ECM. Collagenase was used for isolation of brain endothelial cells (Machi, Kassell, & Scheld, 1990; Wolburg et al., 1994). Combination of collagenase, hyaluronidase and DNAse was used for studying human brain tumor cells (Hussein et al., 2011; Veshchev et al., 2016). Despite the high viability of lymphocytes, collagenase removes the CD4 surface marker (Migliori et al., 2014).

Hyaluronidase

Hyaluronidase is an enzyme degradating hyaluronan, a structural proteoglycan in the ECM, and is standardly used in the mixture of enzymes with collagenase, DNAse and neutral protease. This combination of enzymes was used in several papers associated with brain tissue dissociation. However, cell suspension treated with this mixture of enzymes has a high level of cell clumps, debris and released DNA fragments (Veshchev et al., 2016).

Preparing a mixture of various enzymes in appropriate proportion is difficult and may add on to other possible issues present in obtaining proper single cell suspension.

Accutase

Accutase is a mixture of protelytic and collagenolytic enzymes with DNAse activity which preserves antigen expression and maintains intact cell morphology with high cell yield. Benefit of using only Accutase is a relatively higher comapared to the coctail of several enzymes (Bajpai, Lesperance, Kim, & Terskikh, 2008; Robinson, Rodgers, Goings, & Miller, 2014).

Tumor tissue dissociation kit

Tumor tissue dissociation kit (TTD) has a gentle enzymatic activity optimized for preparation of single cell suspension from tumor tissue for flow cytometry measurement. The kit was used for detection of immune cells in tumor tissue in several studies (Bowman et al., 2016; Pinton et al., 2019).

4.2. Mechanical dissociation

Mechanical tissue dissociation techniques include various nonenzymatical steps like using strainers, tissue chopping, density gradient, trituration strategies or using dissociator machines. Mechanical dissociation is rapid technique with shorter period of cell manipulation, absenting enzymatic incubation. Every manipulative step means stress for cells and may end in reduction of cell viability or cell markers stability. Using nonenzymatical approach may provide insufficient single cell suspension with large group of cells and inconsistent cell yields (Jager et al., 2017). Density gradient method by Percoll and Ficoll is commonly used for isolation of mononuclear cells after passing tissue through strainers, but with lower cell yield than enzymatic dissociation (Dick, Pell, Brew, Foulcher, & Sedgwick, 1997; Neeley & Conley, 1987). This method maintains high viability of CD45 cells and preserves markers of immune cells from dissociated tumor tissue (Migliori et al., 2014).

4.3. Debris removal and erythrocyte lysis

Cell debris, dead cells and erytrocytes provide high background during flow cytometry measurement so it is important to remove them from cell suspension. Cellular debris and clusters can be removed by series of various filters and by density gradient with Debris removal solutions, Ficoll and Percoll. Last step to have high quality single cell suspension is lysis of erythrocytes.

5. Aims of the thesis

We hypothesized that Fibroblast activation protein α (FAP) may play a role in specific infiltration of immune cell subpopulations to the glioma site. We have chosen a flow cytometry for the study of infiltrated immune cells into the tumor microenvironment in immunocompetent syngeneic mouse models of glioblastoma expressing or not expressing FAP. For authentical data by a flow cytometry, it is important to have a proper single cell suspension.

Aim 1: Optimization of dissociation protocol for mouse glioblastoma tissue to study infiltrated immune cells by flow cytometry.

Optimalized dissociation protocol would be applied on glioma tissue of glioma experimental mouse model.

Aim 2: A pilot study of infiltrated immune cells (myeloid populations, CD3 cells, NK cells, Treg) in the microenvironment of glioblastoma flow cytometry measurement in immunocompetent syngeneic mouse model of glioblastoma expressing FAP.

6. Methods

6.1. Cell cultivation

Established mouse glioma cell lines GL261 were obtained from Charles River Laboratories, Inc., (New York, NY, USA). The cells were grown under standard cell culture conditions at 37 °C in the RPMI 1640 medium (Sigma-Aldrich Chemie, Steinheim, Germany), supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich Chemie), under a humidified atmosphere of 5 % CO_2 and 95 % of air.

6.2. Cell counting

Cell suspension was diluted 1:200 (40 µl cell suspension in 8 ml of Coulter Isoton II Diluent) and counted by Coulter Counter Z2 (Beckman Coulter, California, USA). Objects between 10 – 27 µm were counted.

6.3. Stereotactic intracranial implantation

The experimental use of animals was approved by The Commission for Animal Welfare of the First Faculty of Medicine of Charles University and the Ministry of Education, Youth and Sports according to animal protection laws.

Male mice C57BL/6J WT and C57BL/6J FAP-/- (The Jackson Laboratory, Bar Harbor, ME), 6-8 weeks old weighing 25 g were used in experiment. Experimental animals were kept in The Center for experimental biomodels 1st Faculty of Medicine, Charles University. Mice were fully anesthetized with an intramuscular injection of Ketamin (100 mg/kg) a Xylazin (20 mg/kg) before the surgery. The head was shaved, disinfected with Betadine and medial skin cut in lenght 5 mm was performed for following cephalotripsy. The mouse was placed on stereotaxic device (Stoelting, Wood Dale, IL) and head was fitted into the adapter and fixed. The cephalotripsy was performed by a 0,4 mm burr drill with coordinations of 3 mm anteriorly from bregma suture and then 2,5 mm laterally from the sagital suture. The total volume of 5 µl (2000 cells/µl RPMI 1640) of Gl261 cell suspension was injected with 10 µl Hamilton syringe in depth 3 mm in posterior hippocampus during 5 min (1µl/1min) (Fig.1). The Hamilton syringe was left in brain for 3 min after which it was slowly withdrawn during another 2

min. After removing the Hamilton syringe, the cephalotripsycal hole was immediately covered by sterile bone wax. The edges of the incision were reapproximated with standard sewing atraumatic set (Baumann, Dorsey, Benci, Joh, & Kao, 2012). All the surgical interventions were done according to aseptic and antiseptic methods.

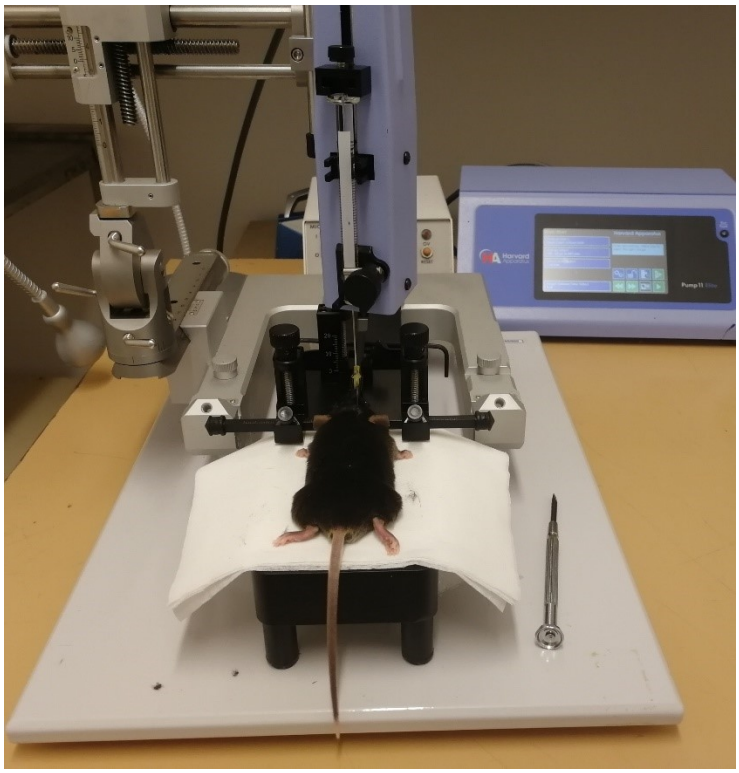


Figure 1: Stereotactic intracranial implantation

6.4. Transcardial perfusion

Mice were fully anesthetized, placed and fixed dorsally on dissection board. By using iris scissors, lateral incision was made through the abdominal wall and rib cage at both sides in caudocranial direction to expose the liver and the heart. The transcardial perfusion method was done by 10 ml syringe and 20 G needle filled with Phosphate buffer, pH 7,4 (PBS) by inserting injeciton into the apex of left ventricle and incising the right atrium with iris scissors. The transcardial perfusion was started off immediatly with flow rate 10 ml/min (Gregory J Baker, Castro, & Lowenstein, 2015).

6.5. Isolation of peritoneal macrophages

Mice were fully anesthetized and fixed dorsally placed on dissection. We made a small incision in the centre of abdomen and firmly peeled the skin off to expose the peritoneal wall. We inserted the 20 G needle to peritoneal membrane, injected 10 ml of PBS into the peritoneal cavity and massaged the abdomen for 10 – 15 seconds. The peritoneal fluid was carefully withdrawn avoiding organs and fat which can clog the needle.

6.6. Retro-orbital blood collection in mouse

Mouse was fully anesthetized. The mouse neck was gently scruffed, which made the eye bulge. A capillary tube with 20 µl of Trypsin-ethylenediaminetetraacetic acid (EDTA) was inserted in venous sinus and blood could flow. We collected 200 µl of blood in 600 µl eppendorfs.

6.7. Dissociation of tissue into single cell suspension

Tissue (spleen, right brain hemisohere) was cut into small 2 – 3 mm pieces in Petri dish. 5 ml of Dulbecco's modified eagle's medium F12 (DMEM) (Sigma-Aldrich Chemie) + 10 % FBS was added to minced tissue for protocol A or 5 ml of accutase (Sigma-Aldrich Chemie) for protocol B and incubated in 37 °C for 30 min. Minced tissue meant to be processed by gentleMACS™ Dissociator (Miltenyi Biotec GmbH, Germany) was transfered to C tubes (Miltenyi Biotec) with 4,7 ml of DMEM and Tumor tissue dissociation kit (TTD) (Miltenyi Biotec) for protocol C, 5 ml of DMEM + 10 % FBS was added for protocol D and tissue was dissociated by program Brain_03 (60 s) followed by incubation in 37 °C for 30 min. After incubation, dissociated tissue was centrifuged at 161 g in 4 °C for 5 min. Supernatant was discarded and pellet was resuspended in Staining buffer (RD systems, Minneapolis, CA, USA) and cells were ready for staining.

6.8. Debris removal

Pellet of cells obtained after centrifugation of filtrated cell suspension was added with PBS to the volume of 6200 µl, then 1800 µl of Debis removal solution (Miltenyi

Biotec) was added. Suspension was mixed by pipetting slowly up and down. The suspension was overlayed very gently with 4 ml of PBS. The sample was centrifuged at 3000 g in 4 °C for 10 min with full acceleration and full brake. After centrifugation, three phases were formed, and top 2 layers were discarded very gently. We added PBS to the volume of 15 ml and inverted the tube for three times. The sample was centrifuged at 1000 g in 4 °C for 10 min. After centrifugation, the supernatant was discarded, and cells were ready for staining. The debris removal protocol was based on manufacturer instructions

6.9. Lysis of erythrocytes

For binding of antibodies with higher specificity and increasing of cell suspension viability we lysed eryhtrocytes by BD FACS™ Lysing Solution (BD, San Jose, CA, USA) which was diluted 1:10 with deionized water for our work. We added diluted 3 ml per 100 µl of cell suspension and incubate for 6 min in 4 °C. Erythrocytes are lysed under gentle hypotonic conditions while leukocytes stayed preserved. The erythrocyte lysis protocol was based on manufacturer instructions.

6.10. Fc receptor blocking

Fc receptor is expressed on some subpopulations of immune cells and and may bind the Fab part of antibodies. Fc receptor blocking reagent (Miltenyi Biotec) blocks Fc receptors on immune cells and increases the specificity of antibodies. We added 10 µl of Fc receptor blocking reagent to 90 µl of cell suspension and incubated for 10 min in 4 °C. After incubation, the sample was centrifuged at 161 g in 4 °C for 4 min. The sample was ready for staining. The Fc receptor blocking protocol was based on manufacturer instructions.

6.11. Data acquisition

Data for a flow cytometer BD FACS Verse were acquired according to the parameters shown in Tab.1.

Tab.1 BD FACS Verse cytometer characteristics

Laser	Emission filter	Fluorochrome
Violet 405 nm	448/45	BD Horizon V450
	528/45	BD Horizon V500
Blue 488 nm	488/15	none
	527/32	FITC
	585/42	PE
	700/54	PerCP
	783/56	PE-Cy7
Red 640 nm	660/10	APC
	783/56	APC-Cy7

6.12. Panel of antibodies

The list of antibodies used in this study and their specifications are shown in Tab.2.

Tab.2 Characterization of antibodies

Laser	Antibody	Fluorochrome	Clone	Isotype	Supplier
Violet 405 nm	LIVE/DEAD™ Fixable Violet Kit	V450			Invitrogen
Blue 488 nm	CD45	PerCP	EM-05	Rat IgG	Exbio
	CD3	PE-Cy7	145-2C11	Hamster IgG	Exbio
	CD8a	PE	53-6.7	Rat IgG2a kappa	Exbio
	CD4	FITC	GK1.5	Rat IgG2b	Exbio
	FoxP3	PE	1954C	Rabbit IgG	RD systems
	Helios	PE	22F6	Hamster IgG	RD systems
	CD335	PE	29A1.4	Rat IgG2a kappa	RD systems
Red 640 nm	F4/80	APC	BM8	Rat IgG2a kappa	RD systems

	CD25	APC	280406	Rat IgG2A	RD systems

Three panels for detection of immune subpopukations by flow cytometry were developed (Tab.3). Immune cells were identified by surface and intracellular positive and negative markers.

Tab.3 Panels of antibodies for immune subpopulations detection

Panels	Cell populations	Markers	Fluorochrome
Panel 1	**Lymphocytes** (CD45+CD3+CD4+CD8+)	CD45	PerCP
		CD3	PE-Cy7
		CD8a	PE
		CD4	FITC
	Myeloid populations (CD45+F4/80+CD3-)	F4/80	APC
Panel 2	**Treg** (CD4+CD25++FoxP3+) (CD4+CD25++Helios+)	CD4	FITC
		CD25	APC
		FoxP3	PE
		Helios	PE
Panel 3	**NK cells** (CD45+CD335+CD3-)	CD45	PerCP
		CD3	PE-Cy7
		CD335	PE

6.13. Dye to detect dead cells

The LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit (Invitrogen, California, USA) was used for determination of cell viability for flow cytometry by channel 448/45 and fluorochrome V450. The dye reacts with intracellular and extracellular amines. In the viable cells, the staining is limited only to extracellular amines. Cells with compromised membranes are stained also intracellulary so they are more positive the dye staining.

For gating strategy of viable cells, we did dead cells control for LIVE/DEAD Fixable Violet Dead Cell Stain Kit, when the spleen was heated in 60 °C for 20 minutes (based on manufacturer instructions of dead cells control). Positive control of dead cells

was used for gating strategy of viable CD45+ leukocytes in dissociated spleen and brain by different protocols (Fig.2).

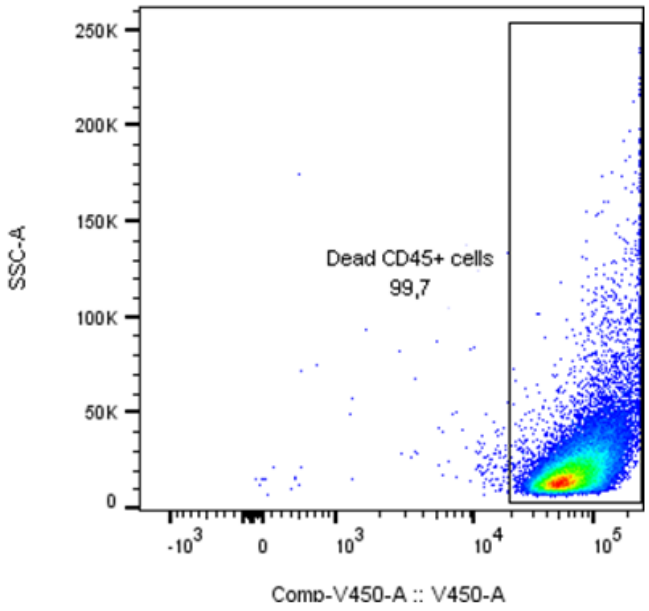


Figure 2: Dead cells control for LIVE/DEAD Fixable Violet Dead Cell Stain Kit

6.14. Staining for flow cytometry

Immunophenotypization of immune cells was performed by a flow cytometer BD FACSVerse with BD FACSuite Software (BD, San Jose, CA, USA) and FlowJo software (FlowJo LLC, USA) for data evaluation. The samples of 50 µl of cell suspension were incubated for 30 min in dark on ice with anti-CD45-PerCP, anti-CD3-PE-Cy7, anti-CD8a-PE, anti-CD4-FITC (all from Exbio, Vestec, Czech Republic), anti-FoxP3-PE, anti-Helios-PE, anti-CD335-PE, anti-F4/80-APC, anti-CD25-APC (all from RD systems) and The LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit (Invitrogen, Carlsbad, Kalifornia, USA). All antibodies for immune cell markers were used in the titre of 1:20. Dye for dead cells was used in the titre of 1:100. For intracellular staining,

cells were fixed and permeabilized by Foxp3/Transcription factor staining buffer (RD systems). Gating strategies were confirmed by unstained samples, Fluorescence minus one (FMO) controls and backgating.

6.15. Statistical Analysis

The Statistica 12 software (StatSoft, Inc., USA) was used for statistical analyses. The Kruskal–Wallis and Mann Whitney U tests were used as appropriate.

7. Results

7.1. Optimization of protocol for dissociation of mouse glioma tissue to cell suspension for flow cytometry to detect immune cells

Designed protocols for dissociation of mouse glioma tissue to cell suspension suitable for a cytometry measurement were based on results of scientific papers being addressed for viable cell suspension obtained from brain and tumor tissue to study immune cells (Guez-Barber et al., 2012; Migliori et al., 2014; Pösel, Möller, Boltze, Wagner, & Weise, 2016).

The protocols include mechanical dissection of tumor tissue by chopping or by dissociator (gentleMACS™ Dissociator) and enzymatic by accutase (Acc) and Tumor tissue dissociation kit (TTD) or nonenzymatic (DMEM12+FBS) digestion. For handling fragile tumor cells, we used program Brain_03 (60 s) instead of program for tumor tissue. Cell suspensions were incubated for 30 min in 37 °C and filtrated through 40 μm nylon strainers. The cell suspensions obtained from tissue dissociated by different protocols were centrifuged at 161 g in 4 °C for 5 min. The supernatants were discarded, cells were resuspended and prepared for cytometry measurement (Fig.3).

Dissociation protocols were tested for leukocytes viability and surface immune markers stability. The most suitable method based on these parameters was applied for further improvement of cell suspension characteristics. Quality of single cell suspension from dissociated tissue was improved by exclusion of cell debris and lysis of erythrocytes (Tab.4).

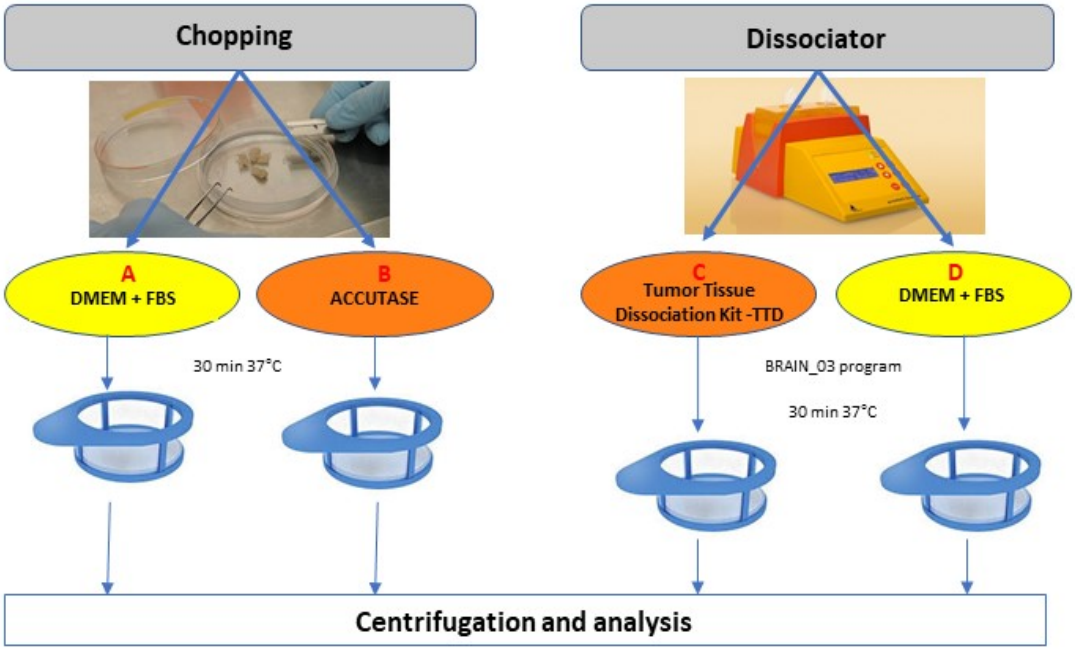


Figure 3: Protocols for dissociation of mouse glioma tissue to detect immune cells by flow cytometry

Tab.4: Measured parameters for optimization of dissociation protocol for mouse glioma tissue

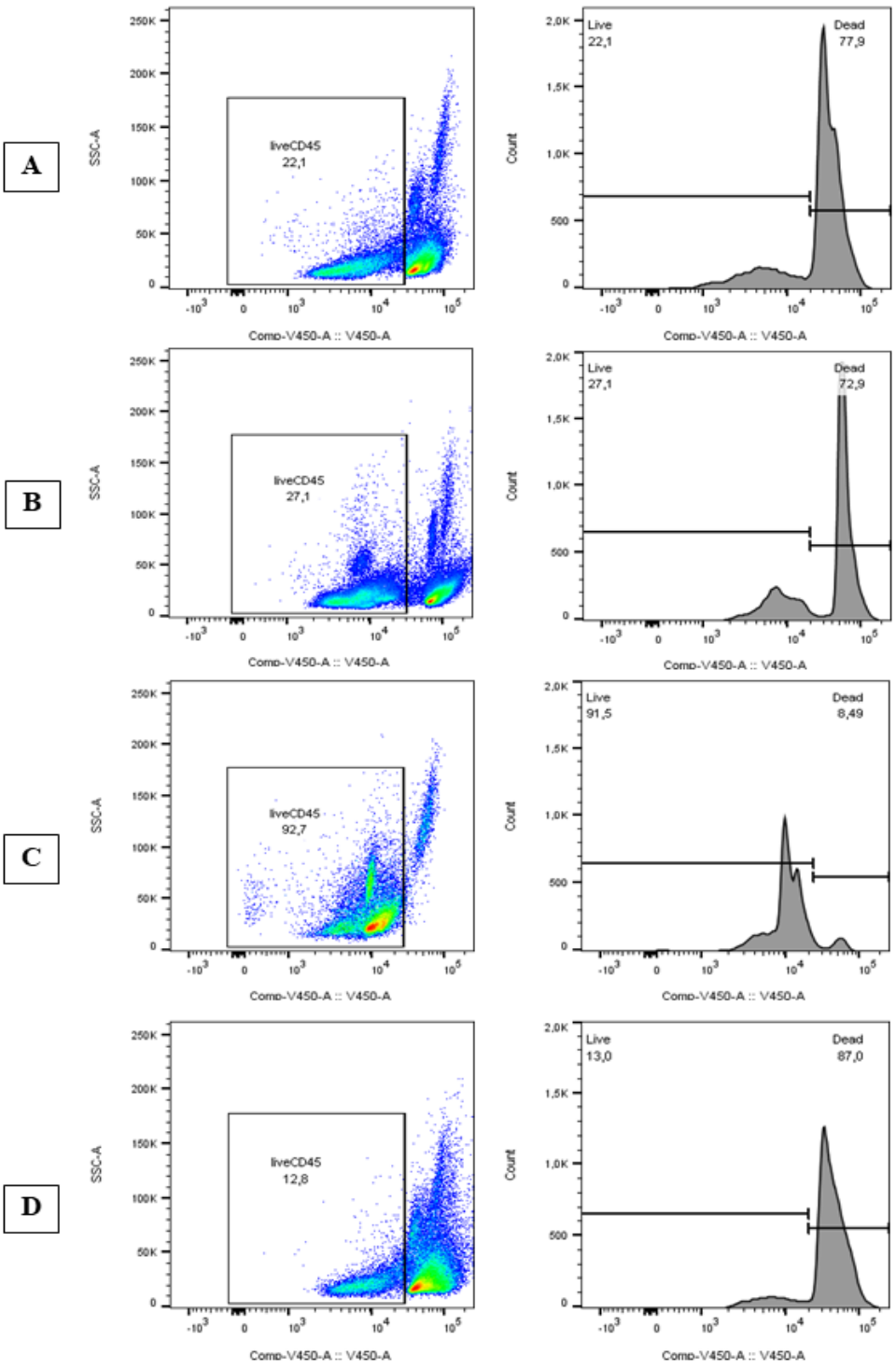
Markers stability	- Testing diggestive effect of used enzymes on surface markers on immune cells - To exclude the effect of markers shedding during dissociation of solid tissue, we tested on body fluids (blood, peritoneal fluid) - Experiment done in triplets
Viability of leukocytes	- Testing of protocols for maintaining maximum viability of leukocytes - Tested on spleen of healthy mouse C57BL/6J - Experiment done in triplets

7.1.1. Comparison of leukocytes viability after tissue dissociation

During the dissociation of the tissue, some viable cells are lost by natural causes. The protocol for preparation the cell suspension form tissue should keep the maximum of cell viability. The viability of cells in glioma may be lower due to necrotic and hypoxic characteristics of tumor. To determine the impact on cells viability by protocol for tissue processing and not by tumor features, the protocols should be tested on healthy tissue. Under physiological conditions, mouse brain lacks some types of immune cell populations, but spleen contains variable populations of leukocytes. To test the viability of CD45+ leukocytes, we dissociated spleens from healthy C57BL/6J mice. The spleens were proceeded by protocols A, B, C and D (see 6.7. Dissociation of tissue into single cell suspension).

After dissociations of spleen tissue, we detected viable leukocytes from CD45+ singlets with excluded debris. The gating strategy for viable leukocytes was based on positive dead cells control (see 6.13. Dye to detect dead cells; Fig.1). Viability of leukocytes was higher in protocols based on enzymatic digestions in comparison with dissociation without enzymes. The highest portion of viable CD45+ leukocytes was obtained from cell suspension created by dissociation of the spleen using the protocol C (Fig.4).

a)


A
B
C
D
SSC-A
Comp-V450-A :: V450-A
liveCD45
22,1
27,1
92,7
12,8
Count
Live
22,1
Dead
77,9
Live
27,1
Dead
72,9
Live
91,5
Dead
8,49
Live
13,0
Dead
87,0

b)

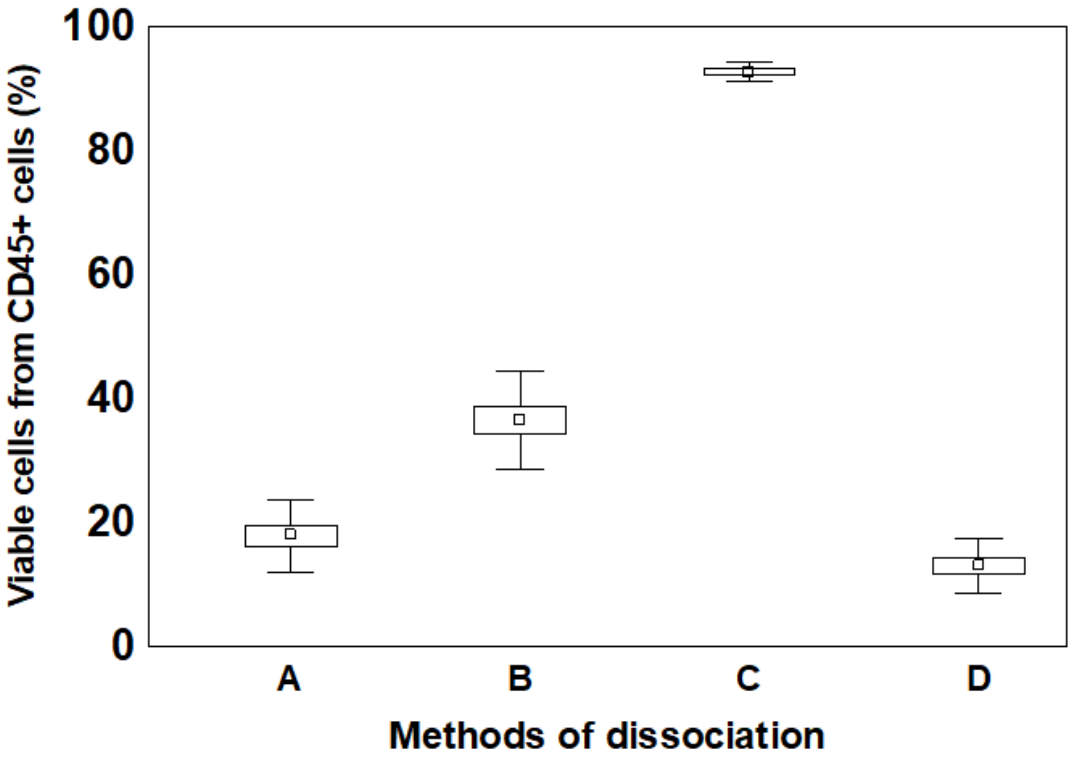


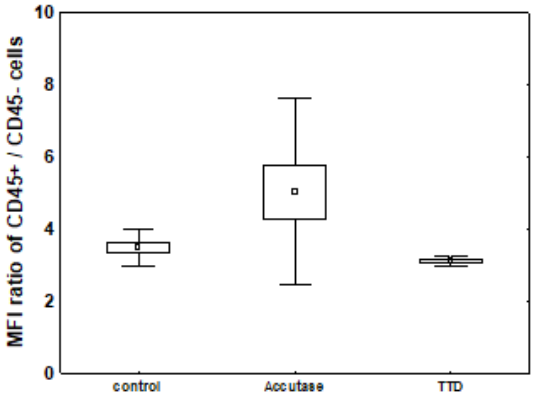
Figure 4: Viability of CD45+ leukocytes in dissociated spleens by protocols A, B, C and D of healthy C57BL/6J mice

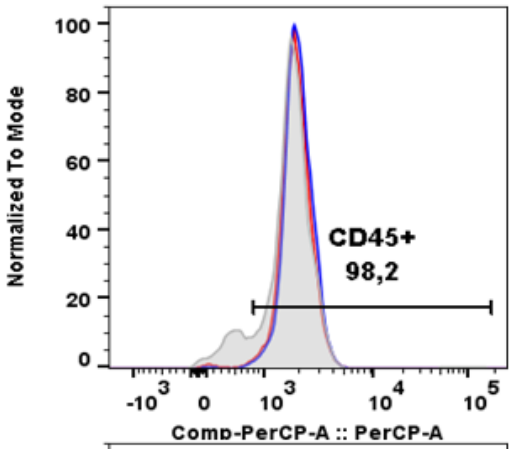
a) *Dotplots and histograms show viable CD45+ leukocytes. Cells were gated on FSC vs SSC channels. Doublets were excluded by FSC-A vs FSC-H channels. Viable cells were gated from CD45+ single cells.* ***b)*** *Percentage mean values of viable CD45+ leukocytes by A, B, C, D dissociation protocols shown in boxplot; Squares: mean values; Boxes: Mean ± SD; Bars: Mean ± 2* SD; A (MV=17,87; SD=2.95); B (MV=36,53; SD=3,95); C (MV=92,67; SD=0,81); D (MV=13; SD=2,23); p value = KW-H(3;12) = 10,2383; p = 0,0166 ±; N=12; Kruskal–Wallis test*

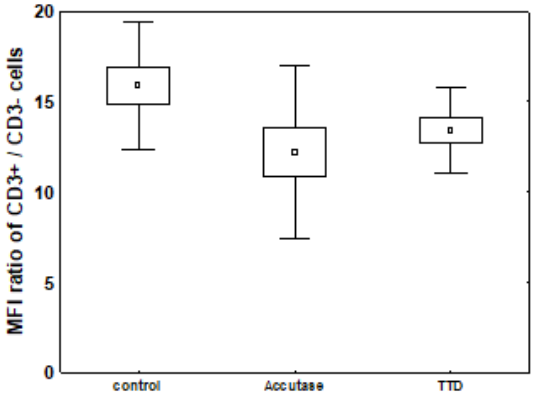
7.1.2. Analysis of surface markers stability on immune cells after enzymatic digestion

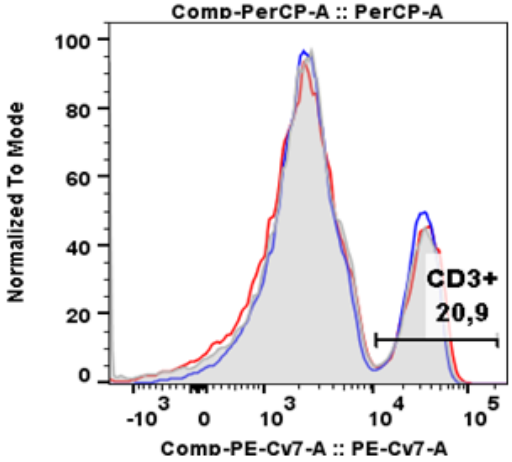
Enzymatic tissue digestion may lead to cleavage of various receptors and antigens on the cell surface. Moreover, the loss of cells markers may be due to the natural cause of degradation of tight junctions and proteins of ECM. We were interested in digesting effect of Tumor Tissue Dissociation kit (TTD) and Accutase (Acc) on

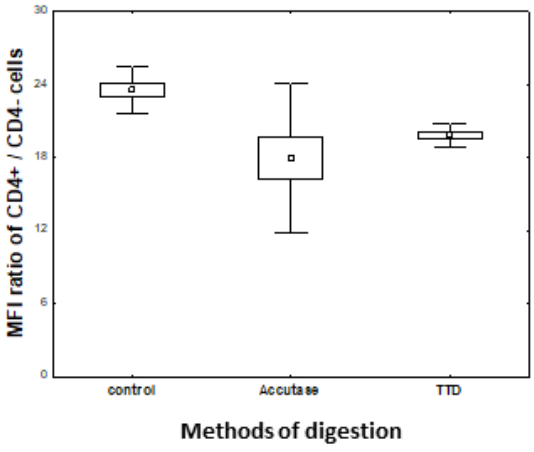
stability of surface markers CD45, CD3, CD4, CD8, CD335 and F4/80 during dissociation of solid brain tumors (Fig.5). The effect of enzymatic treatments on stability of surface markers on immune cells was tested on mouse body fluids (blood for lymphocytes markers, peritoneal fluid for myeloid cells marker). The cells were treated by the same incubation time and temperature as in protocols for dissociation of solid tissue. Afterwards, the samples were measured by flow cytometry. We analyzed the loss of surface markers by measuring ratio of positive and negative median fluorescent intensity (MFI) in samples traeted with TTD, Accutase and nontreated samples. The experiment was done in triplets. The alterations of surface markers in enzymatically treated cells were not statistically significant compared to nontreated cells. The expression of CD335 was slightly higher in cells treated enzymatically both by Accutase and TTD. The same effect was seen for CD45 marker in cells treated by Accutase.

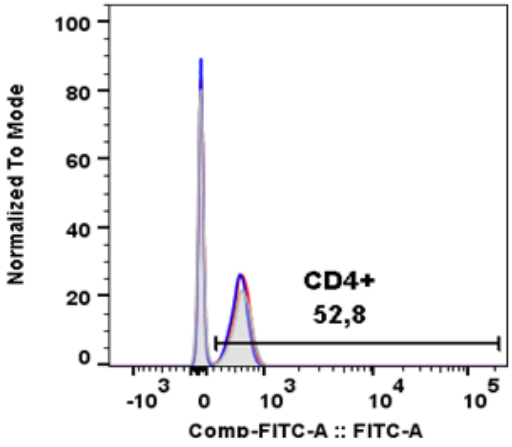

MFI ratio of CD45+ / CD45- cells
10
8
6
4
2
0
control
Accutase
TTD


Normalized To Mode
100
80
60
40
20
0
CD45+
98,2
-10^3
0
10^3
10^4
10^5
Comp-PerCP-A :: PerCP-A


MFI ratio of CD3+ / CD3- cells
20
15
10
5
0
control
Accutase
TTD


Normalized To Mode
100
80
60
40
20
0
CD3+
20,9
-10^3
0
10^3
10^4
10^5
Comp-PE-Cy7-A :: PE-Cy7-A


MFI ratio of CD4+ / CD4- cells
30
24
18
12
6
0
control
Accutase
TTD
Methods of digestion


Normalized To Mode
100
80
60
40
20
0
CD4+
52,8
-10^3
0
10^3
10^4
10^5
Comp-FITC-A :: FITC-A

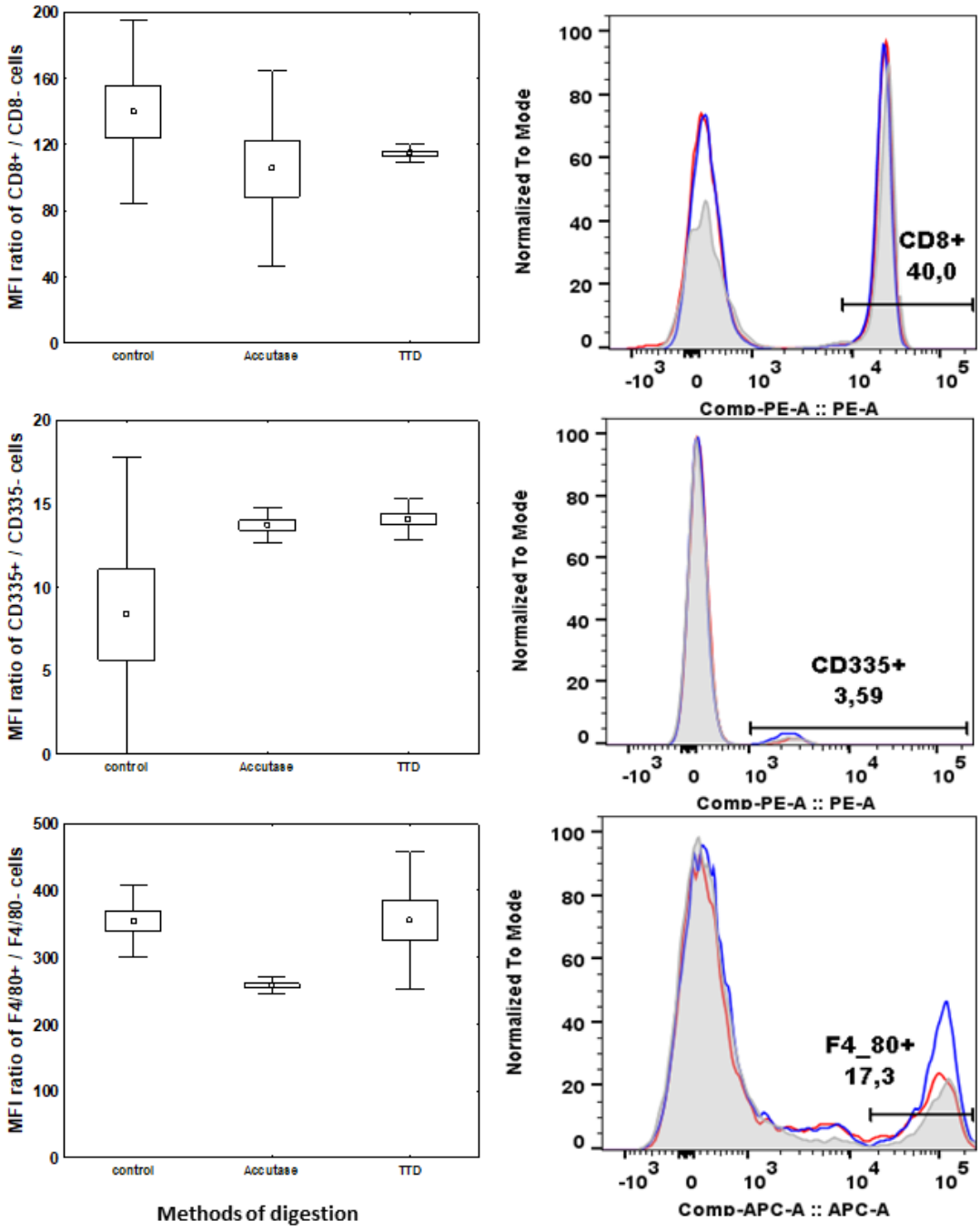

MFI ratio of CD8+ / CD8- cells
control
Accutase
TTD
Normalized To Mode
CD8+
40,0
Comp-PE-A :: PE-A
MFI ratio of CD335+ / CD335- cells
CD335+
3,59
MFI ratio of F4/80+ / F4/80- cells
F4_80+
17,3
Comp-APC-A :: APC-A
Methods of digestion

Figure 5: Surface markers stability in enzymatically treated samples in comparison with non-treated samples

Acc – accutase, TTD – tumor tissue dissociation kit, control – non-treated samples, MFI – mean fluorescence intensity ratio, Histograms of flow cytometry measurement; Percentage mean values of MFI CD45, CD3, CD4, CD8, CD335, F4/80 by A, B, C, D dissociation protocols shown in boxplots: Mean ± SD; Bars: Mean ± 2 SD; CD45 (KW-H=2,7556; p=0,2521); CD3 (KW-H=2,4889; p=0,2881); CD4 (KW-H=5,535; p=0,0628); CD8 (KW-H=3,2889; p=0,1931); CD335 (KW-H=2,7556; p=0,2521); F4/80 (KW-H=5,4222; p=0,0665); N=12; Kruskal–Wallis test*

7.1.3. Analysis of debris removal effect on cell suspension quality

Glioma tissue contains necrotic lessions with dead cells. Moreover, the dissection of tissue and enzymatic digestions may lead to apoptosis and injury of the cells. Dead and damaged cells release intracellular compartments to extracellular environment. Released intracellular compartments cause aggregating of cells. Debris and cell aggregates in cell suspension may be a problem for acquiring a reliable data by flow cytometry. In order to get more accurate results, we used Debris removal solution MACS Miltenyi for excluding debris and dead cells.

The pictures taken by a light and fluorescent micropscope are showing the composition of cell suspension acquired from dissociated brain tissue by protocol C. The cells that were not clustered had nucleus stained with Hoechst. In the Fig.6, a) cell suspension contains viable cells and debris, b) is showing excluded debris from cell suspension as control for not losing viable cells and c) we can see viable cells that are not aggregated and are with much less debris. After the exclusion of debris, dead cells and cell aggregates, the sample had higher qualitative properties of a single cell suspension.

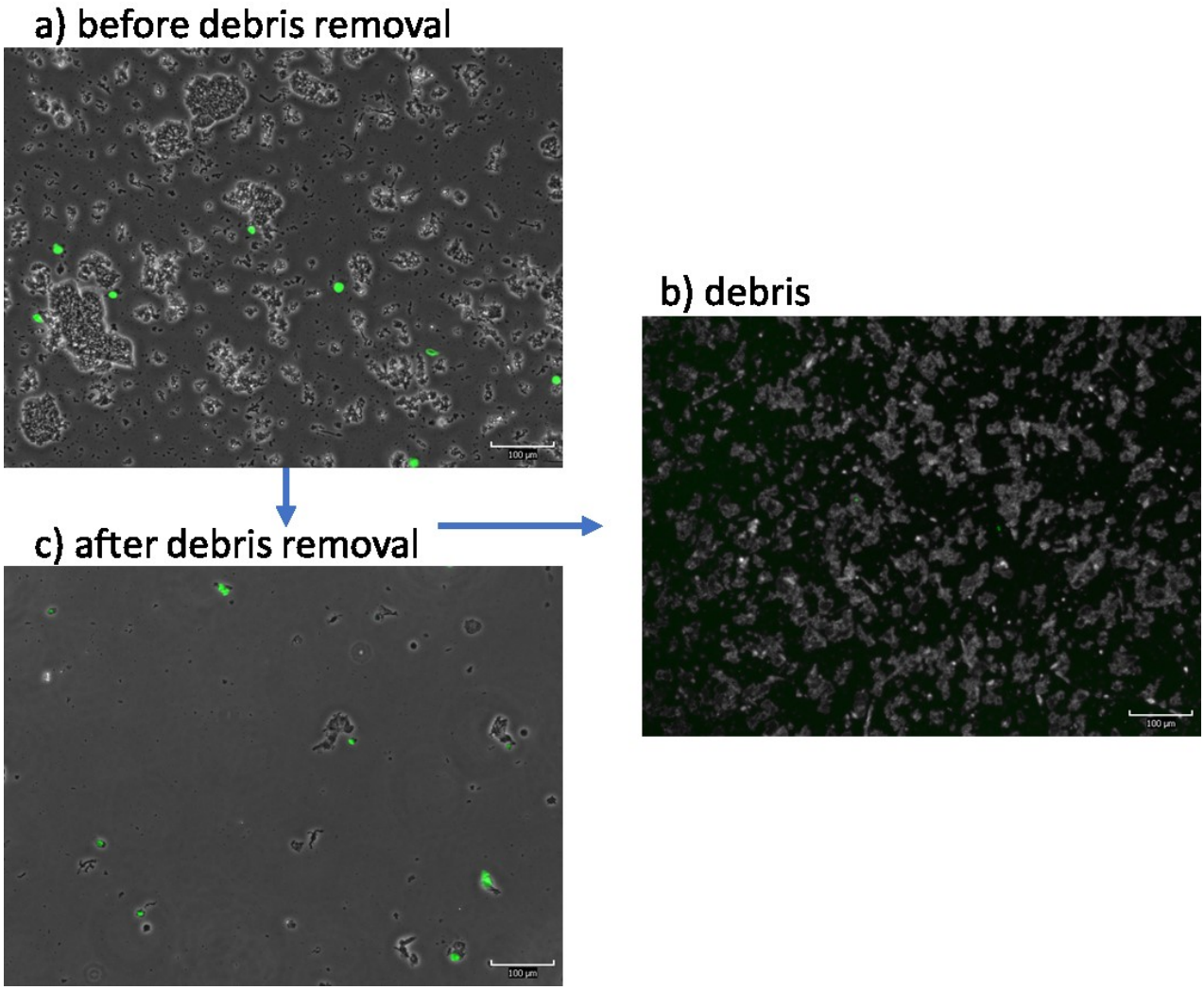


Figure 6: Exclution of debris in cell suspension

7.2. Characterization of tumor yield in immunocompetent syngeneic mouse model of glioblastoma

Mouse glioma cell line was implanted intracranially into right cerebral hemisphere of immunocompetent syngeneic mouse model of glioblastoma (see 6.3. Stereotactic intracranial implantation). Mice bearing glioma were harvested after 4-5 weeks, after observation the symptoms related to the tumor growth (behavioral characteristics, loss of fur and body weight, arched back, unsteady posture). Mouse glioma is characteristic for high angiogenesis, compacted growth and non infiltrative tumor cells to the surrounding tissue (Fig.7). The tumor tissue was used for testing of leukocytes viability by different dissociation protocols to confirm our results of the highest viable leukocytes obtained by the dissociation protocol C. The optimilized

protocol was applied on mouse glioma located in right hemisphere to study infiltrated immune cells.

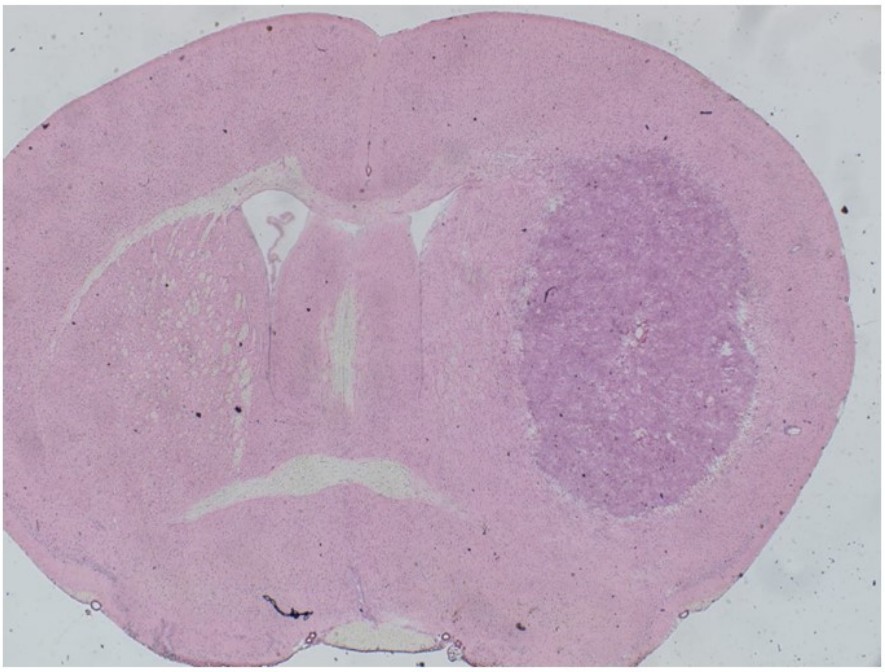


Figure 7: Hematoxylin-eosin stained section of mouse brain bearing glioma

7.3. Comparison of leukocytes viability after dissociation of mouse glioma tissue

After testing the dissociation protocols for viability of in spleen from healthy mouse, we needed to confirm the effects on tumor tissue. We validated the leukocytes viability in cell suspension acquired from mouse glioma dissociated by protocols A,B,C and D. Four C57BL/6J male mice aged 6 – 8 weeks were implanted with mouse glioma cells intracranially under stereotactic conditions (one for each protocol). After 4-5 weeks and visible symptoms related to the brain tumor, mice were harvested and brain tumors were collected under ischemic conditions done by transcardial perfusion (see 6.4.Transcardial perfusion). Cell suspensions acquired by different protocols were stained for CD45 marker and dye for dead cells to measure viable leukocytes.

The highest portion of 36,9 % of viable CD45+ cells from all CD45+ cells were detected in cell suspension acquired from brain tumor tissue processed by protocol C

(Fig.8). Nonenzymatic incubation with the use of dissociator obtained only 20,4 % of viable leukocytes. In comparison, tissues dissected by chopping and treated enzymatically with Accutase and nonenzymatically with DMEM+FBS have only 12,5 % and 5,65 % respectively of viable leukocytes from all CD45+ cells.

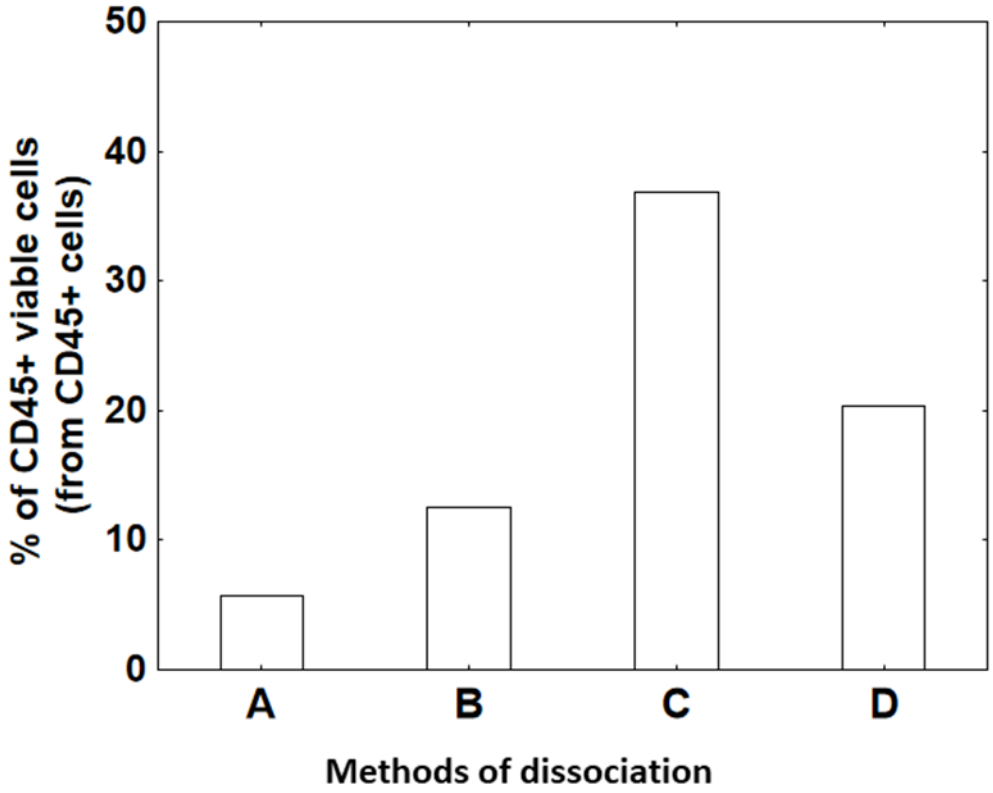


Figure 8: Viability of CD45+ leukocytes in cell suspension from dissociated mouse glioma tissue by various protocols

7.4. Gating Strategy for immune cell populations in mouse glioma tumor

7.4.1. Panel 1 – CD3+ and myeloid cell populations

Cells were gated based on size on FSC vs. SSC channels and debris was excluded. Single cells were identified by FSC-A vs. FSC-H. Viable singlets were gated for CD45+ populations. Panel 1 enabled the counting of T cells (CD45+CD3+CD4+CD8+) and myeloid populations (CD45+F4/80+) (Fig.9).

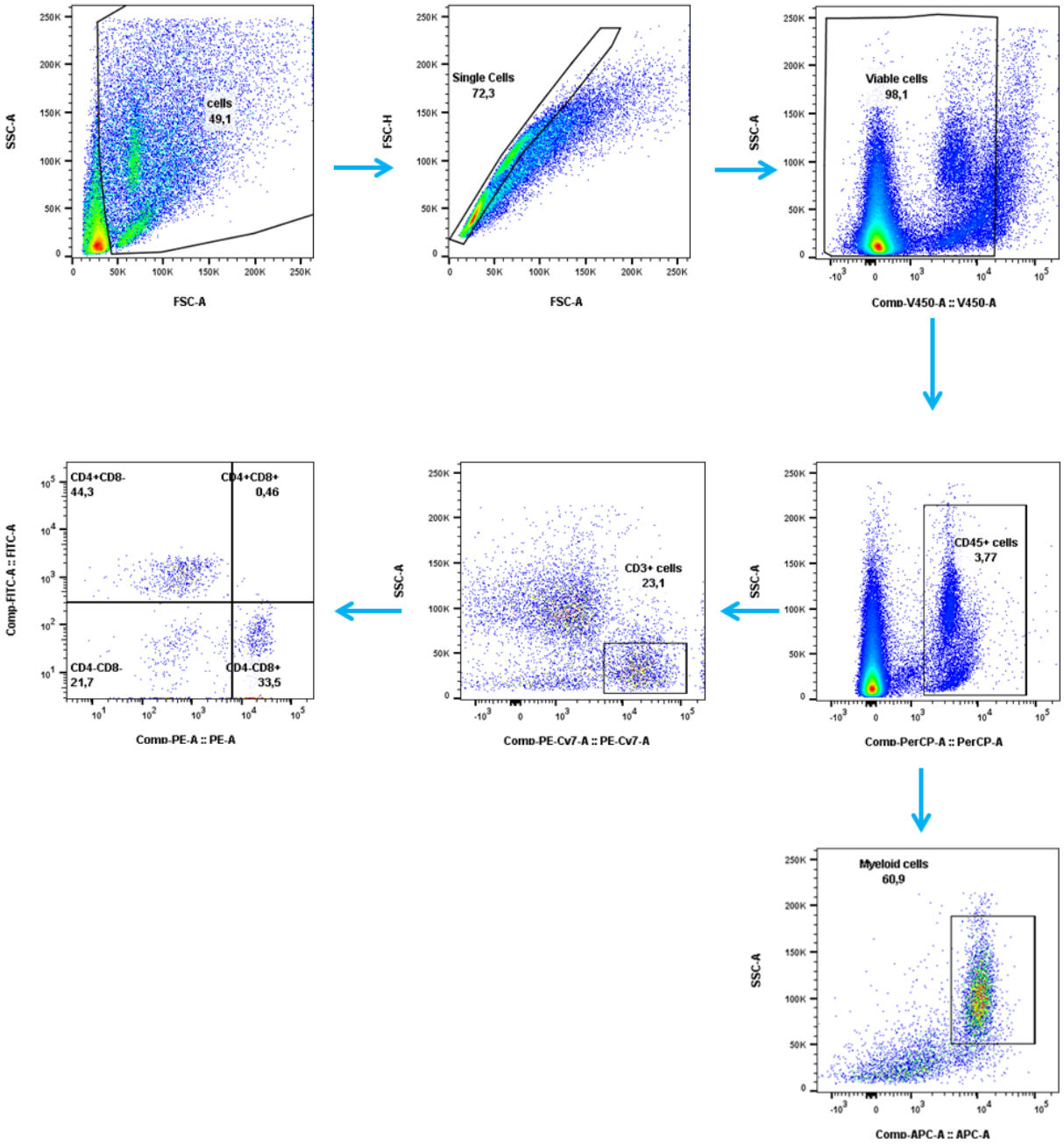


Figure 9: Gating strategy of myeloid cell populations and CD3+ cells in mouse glioma tissue

7.4.2. Panel 2 – Treg

The panel 2 was used for identification of Treg as CD4+CD25++FoxP3+ or Helios+ cell populations. Firstly, cells were stained for surface markers and then fixed, permeabilized and stained for intracellular markers. Longer incubation during fixation, permeabilization and staining may cause cell aggregates seen in FSC vs SSC channel (Fig.10).

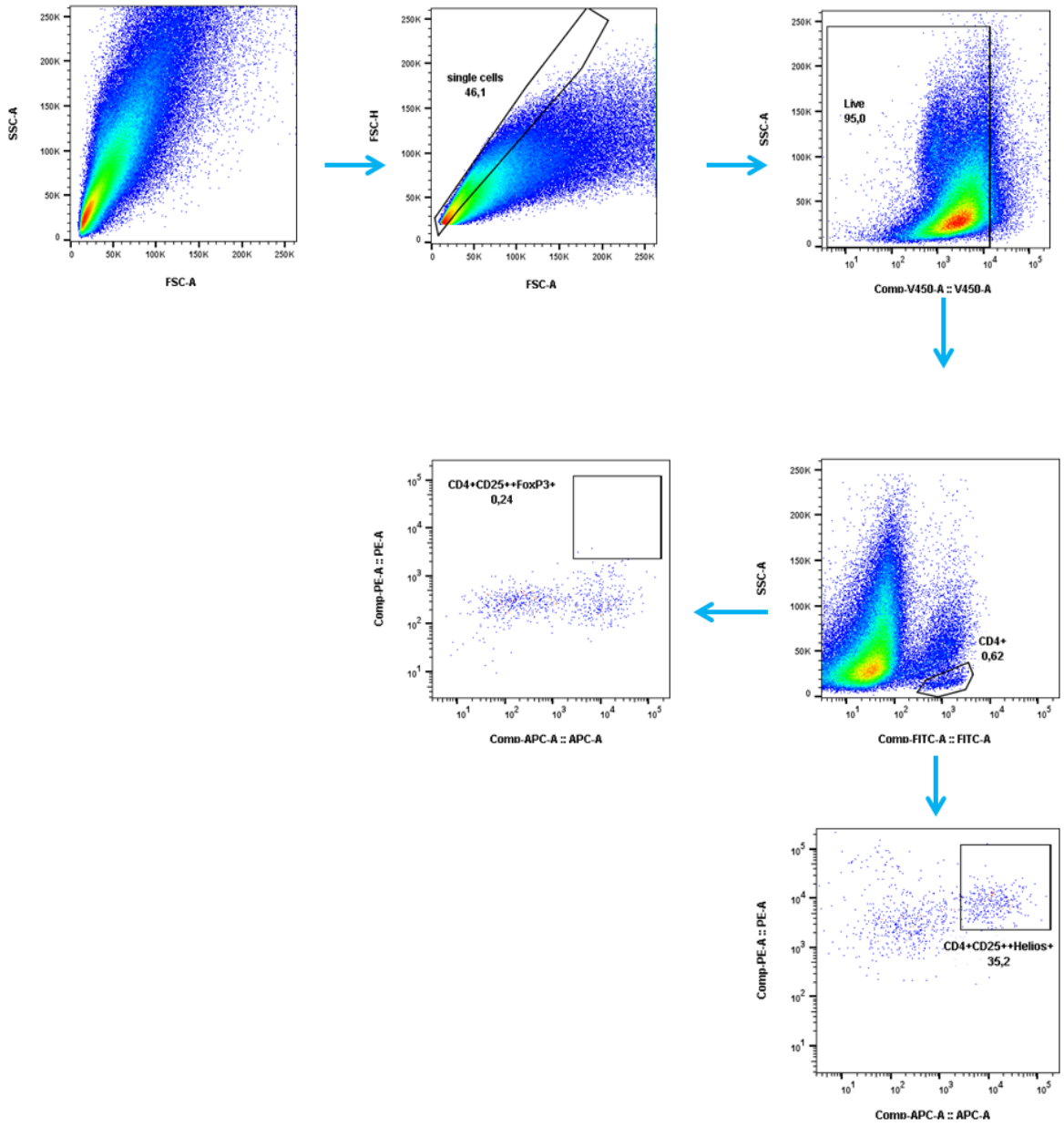


Figure 10: Gating strategy of Treg in mouse glioma tissue

7.4.3. Panel 3 – NK cells

Panel 3 was used for identification of NK cells as CD45+ CD335+ CD3- population. Firstly, we excluded cell debris. Lymphocytes were gated based on morphology on FSC vs. SSC channel. Single cells were identified by FSC-A vs. FSC-W. CD45+ population was identified according to negative control of unstained sample. Viable NK cells were identified as CD3- negative population and positive for CD335 marker (Fig.11).

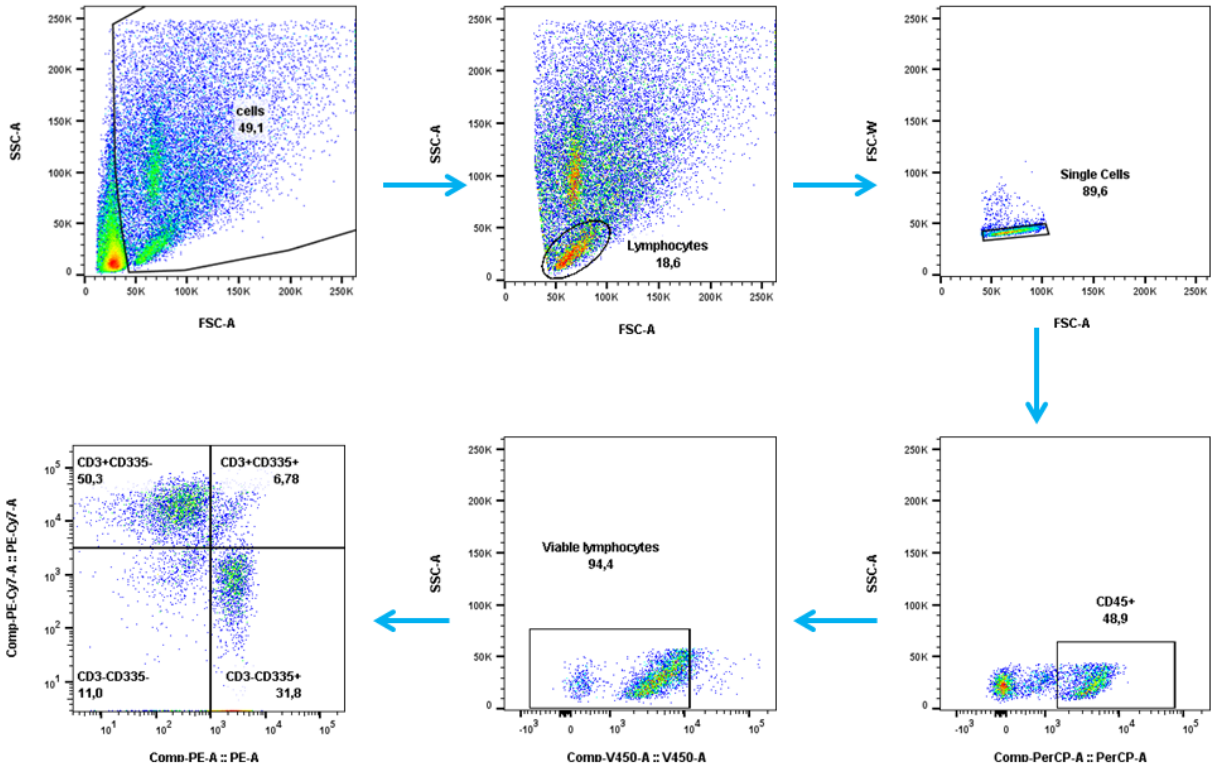


Figure 11: Gating strategy for NK cells in mouse glioma tissue

7.5. Detection of immune cells in syngeneic mouse model of glioblastoma with/without expression of FAP

The protocol C was considered as the most suitable method for dissociation tissue into single cell suspension, with the highest viable leukocytes yield and preservation of surface markers on immune cells. The cell suspension was improved by debris removal. The protocol was applied on mouse glioma tissue to detect infiltration of immune cells into tumor microenvironment in dependance of FAP expression in syngeneic mouse model of glioblastoma. Three C57BL/6J WT and three C57BL/6J FAP-/- male mice aged 6-8 weeks were implanted intracranially with mouse glioma cells GL261. Brain tumors grew for 4-5 weeks. Afterwards, the tumors were harvested and dissociated into single cell suspension and stained for immune cell markers and measured by flow cytometry. The portion of F4/80, CD3 and CD335 cells were gated from viable CD45 singlets. Cells CD4 and CD8 were identified from viable CD3 single cells.

The differences in portion of infiltrated immune cells between C57BL/6J WT and C57BL/6J FAP-/- were not statistically significant (Fig.12).

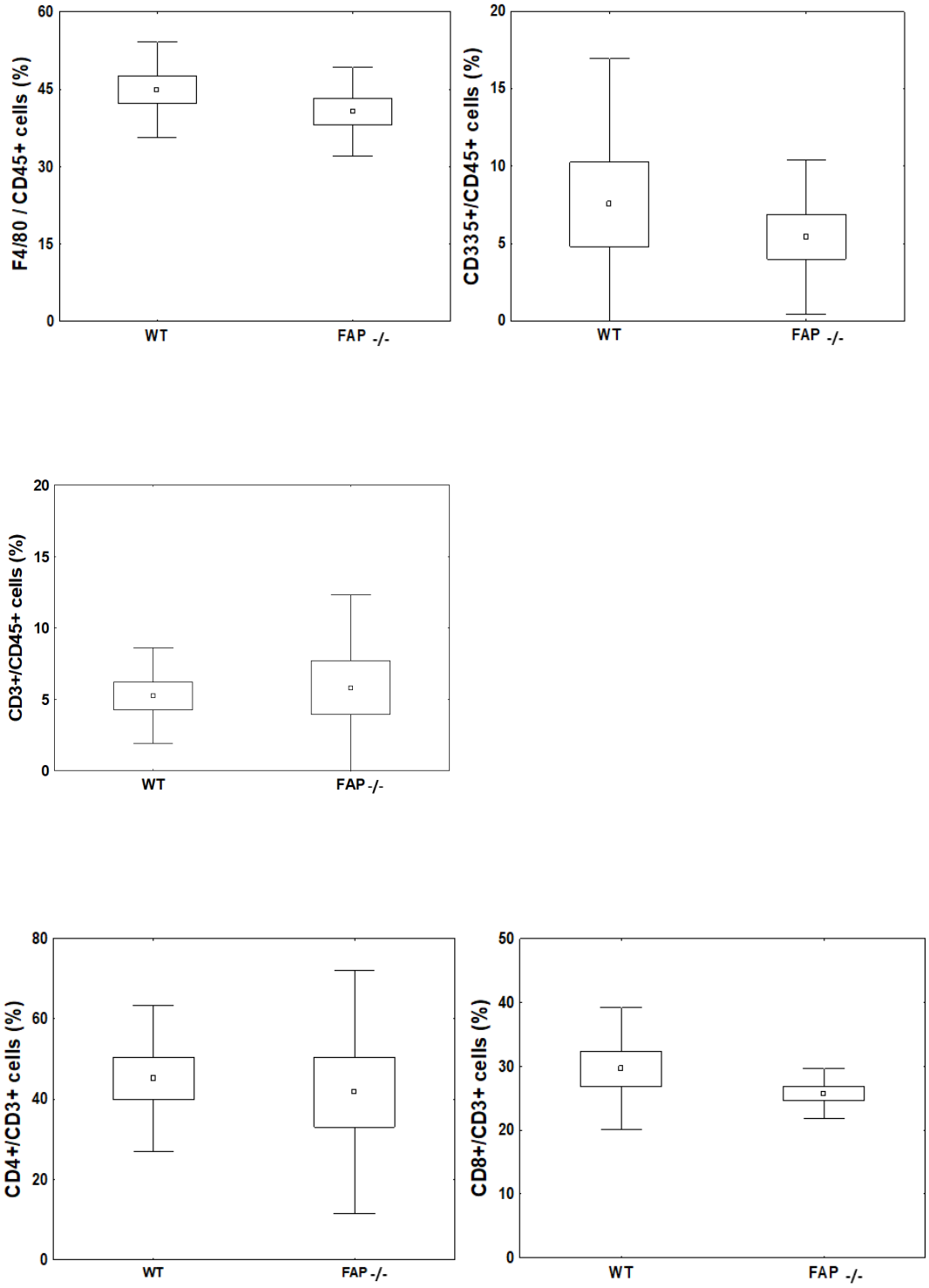


Figure 12: Percentage of studied cell populations in C57BL/6J WT and C57BL/6J FAP -/- glioblastoma mouse models *Preliminary results did not show any statistical differences (using Mann Whitney U test) between* C57BL/6J *WT and* C57BL/6J *FAP -/- mice.*

8. Discussion

Ongoing clinical trails are based on targeting not only cancer cells but also immune and stromal cells in the tumor microenvironment. Immunomodulating effect of immunotherapy helps immune system to fight cancer (reviewed in Farkona, Diamandis, & Blasutig, 2016). Fibroblast activation rotein α is the candidate for targeting stromal cells in anticancer therapy (Cai et al., 2013; N. Liu et al., 2015). A better understanding of cell components in tumor microenvironment and their interactions is important for novel strategies of anticancer treatments.

Flow cytometry allows analysis of large number of cells in single cell suspension at short time with relative precision. The goal of tumor tissue dissociation into single cell suspension should be to preserve maximum of cells viability and phenotype of analyzed cells.

The study describes the various dissociation methods, including enzymatic and nonenzymatic digestion and mechanical tissue dissection by dissociator or chopping for preparation of mouse glioma homogenates for flow cytometry and their limitations for leukocytes viability and markers stability. The optimized dissociation protocol was then applied on mouse glioma tissue for a pilot study of infiltrated immune cells and the tumor microenvironment expressing FAP. Besides the results, a significant part of the thesis was to acquire skills in experimental methods using mouse models in *in vivo* experiments, including intracranial implantation of cell suspension into the mouse under stereotactic conditions (Fig.2) and using flow cytometer as well as evaluation of cytometry data.

We designed four different protocols with enzymatical and nonenzymatical digestion in combination with different ways of tissue dissections. Designed protocols were based on publications of brain or tumor dissociation for immune cells detection. The methods were tested for viable cell yield and preservation of surface immune markers during digestion.

The method should keep the maximum of cells viability during tissue dissociation. To validate the loss of viable cells during dissociation of tissue we needed to test our designed protocols for preparation of single cells suspension on healthy nontumorous tissue. As mentioned above, we wanted to study infiltrated immune cells

in tumor microenvironment, so we tested our designed protocols on healthy spleen from immunocompetent mouse, which have a wider scale of leukocyte populations comparing to a healthy brain. The viability of leukocytes obtained from dissociated healthy spleens digested with enzymes was higher comparing to nonenzymatical digestion. The highest portion of viable cells, about 90 %, were obtained from spleen dissociated by combination of gentleMACS™ dissociator and Tumor tissue dissociation kit for more efficient optimized cell yield. The viability of leukocytes from dissociated spleen tissue by the nonenzymatical digestion was less than 30 % from all CD45+ cells. Nonenzymatical digestion of tissue could be associated with inconsistent cell yields because of insufficient breakdown of tight junctions and proteins of the ECM. Tissue dissociation is associatied with release of intracellular compartments and nucleic acids, responsible for membrane continuum disrupture and change of resting potential. These circumstances lead to the formation of cell aggregates and reduction of cellular viability. Nonenzymatic dissociation method is also not suitable for isolation of neural cells (Jager et al., 2017). The mixture of specific enzymes may be very useful during enzymatic procession in degradation of released intracellular compartments and nucleic acids. However, cell suspension of dissociated breast tumor tissue had increased cell viability and cell recovery when using mechanical dissociation by dissociator without enzymes (Migliori et al., 2014). The structure of tissue may be the reason of different results of leukocytes viability in cell suspension form dissociated tissue even with the same dissociation protocol. Tumor tissue like glioblastoma is more difficult to handle than nontumorous tissue because of wide necrotic areas and high angiogenesis, using of enzymatic digestion may be controversial. However, the leukocytes viability from tumor tissue obtained by dissociation with dissociator and Tumor Tissue Dissociation kit was still the highest (almost 40 % from all CD45+ cells). Using dissociator in dissociation of tumor tissue without enzymatic incubation lead to only 20 % of viable leukocytes. In comparison, tissue dissected by chopping and treated enzymatically and nonenzymatically have less than 15 % of viable leukocytes from all CD45+ cells.

Degradation of proteins of extracellular matrix and cell junctions during tissue dissociation may lead to profound effects on surface marker expression and even their enzymatic cleavage. Lymphocytes tend to lose CD4 markers during enzymatic digestion (Migliori et al., 2014). Loss of markers of our interest may lead to not-authentical data. For direct validation of enzymatic effect on surface markers stability, we incubated

enzymes (accutase, Tumor tissue dissociation kit) with single cell suspensions of body fluids (peritoneal fluids, blood). Enzymatic treatments of single cell suspension with accutase and Tumor tissue dissociation kit have no significant effect on surface expression of most tested molecules, but surprisingly, comaperd to the untreated control, Tumor tumor tissue dissociation kit and accutase induced surface expression of CD335 marker. The exposure to certain enzymes may promote phenotype alterations. Higher expression of typical markers of myeloid cells may be induced by enzymatic treatment. Various enzymes may affect recovery of surface molecules on immune cells in *in vitro* culture with different effect (Autengruber, Gereke, Hansen, Hennig, & Bruder, 2012).

We considered the protocol with using dissociator and Tumor tissue dissociation kit as the most suitable method for dissociation of mouse glioma for a flow cytometry measurement. This protocol preserved the highest viability of leukocytes and and surface immune markers were not altered during enzymatic digestion. For further experiments, we worked only with the protocol using dissociator and Tumor tissue dissociation kit for tissue dissociation.

During the process of tissue dissociation, some cells are harmed and die which is associated with release of nucleic acid and intracellular compartmens and formation of debris from dead cells. Cell suspension with debris is a problem for a precise flow cytometry measurement. We tested the effect of debris removal by gradient density on dissociated healthy mouse brain tissue. Exclution of debris removal improved properties of single cell suspension. Removed cellular debris showed no compacted cells.

After optimization of protocol for dissociation, we processed the mouse glioma tissue for a flow cytometry measurement. When we were able to acquire a proper single cell suspension from mouse glioma tissue, we developed and tested panel of antibodies for immune cells detection. For gating strategy, we used unstained controls, fluorescence minus one control and backgating. We were able to dectect lymphocyte (CD3+ cells, CD335+ NK cells,) and myeloid cells (F4/80). We identified viable CD3+ lymphocytes (CD4, CD8), NK cells (CD335+CD3-) and myeloid cell populations (F4/80+, CD3-).

Regulatory T-cells (Treg) were identified by their surface markers (CD4+, CD25+) and intracellular markers (FoxP3+, Helios). Longer incubation time during

fixation and permeabilization of Treg in cell suspension caused cell aggreagation and single cell suspension lost its properties suitable for flow cytometry measurement. Using only positive surface markers CD4 and CD25 and negative marker CD127 is more sutable for further identification of Treg in the cell suspension obtained from dissociated tumor tissue.

For describing of the role of FAP in specific infiltration of immune cell subpopulations in glioblastoma microenvironment pilote results from the experiment with 3 C57BL/6J WT and 3 C57BL/6J FAP-/- male mice intracranially implanted with mouse glioma cells GL261 were shown. Analyzed tumors showed no statistically significant differences between frequency of infiltrated immune cells F/4/80, CD3, CD4, CD8 and CD335 in mouse with and without expression of FAP. However, the variabilities in one infiltrated immune population were wide. The experiment of implanted glioma cells in syngeneic mouse model of glioblastoma brought several technical problems. The different time of harvesting tumors and their different size might cause the variabilities in one infiltrated immune population. For more reliable data, the experiment should be repeated.

Tumors have higher tendency of lymph node metastasis and recurrence when FAP is overexpressed (F. Liu et al., 2015). Depletion of FAP leads to restriction of tumor growth associated with change of antitumor immune response. Expression of FAP promotes CCL2 production and STAT3 expression which correspond to a shift in Th2 immune response (X. Yang et al., 2016). High FAP expression also correlates with reduced infiltration of CD3 cells in tumor microenvironment (Chen, Qiu, Wang, & He, 2017).

Notably, FAP is important for tumor growth and progression but its role in immunosuppression in GBM is still unknown and need to be elucitated. For the limited expression in normal tissue, overexpression in tumor milieu together with protumorigenic effects suggest that FAP is a prospective candidate for new therapeutical approaches in treatment for GBM. There are already ongoing clinical studies of therapies targeting FAP in hepatic metastases, colorectal cancer and non-small cell lung cancer and other cancers (Narra et al., 2007; Scott et al., 2003; Soerensen et al., 2018).

9. Conclusion

Protocol for glioma tissue dissociation and preparation of single cell suspension suitable for flow cytometry analysis was optimized. The established protocol keeps maximal viability of immune cells and condition of extracellular surface immune cell markers for further analysis.

A pilot study of infiltrated immune cells (myeloid populations, CD3 cells, NK cells, Tregs) in orthotopic immunocompetent mouse model was performed and optimalized for further study of FAP effect in local immunosuppression in glioblastoma tumor microenviroment.

Methodologies developed in this diploma thesis will be used for further studies evaluating the role of FAP in glioblastoma immunosuppression. Deeper insight into glioblastoma microenvironment will be beneficial for searching of novel therapeutic strategies.

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