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Reciprocal interaction of melanoma brain metastasis with cells of the tumor microenvironment

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Abstrakt (Deutsch):

Hintergrund: Das Maligne Melanom gehört zu den tödlichsten Hauttumoren. Wenn ein Melanom nicht frühzeitig erkannt und behandelt wird, weist es eine hohe Metastasierungsrate auf. Im fortgeschrittenen Stadium entwickeln ca. 40-50% der betroffenen Patienten Hirnmetastasen. In der Tumormikroumgebung von Hirnmetastasen gehen Melanomzellen komplexe Interaktionen mit ortsansässigen Zellen ein, unter anderem mit Astrozyten. Wenn Astrozyten mit Melanomzellen interagieren, können sie beispielsweise durch die Sezernierung von Zytokinen tumorfördernde Eigenschaften entwickeln. Es konnte bereits gezeigt werden, dass Hirnmetastasenzellen des Malignen Melanoms genetische Veränderungen hinsichtlich Migration, Intra- / Extravasation und Differenzierungsgrad aufweisen und somit den Metastasierungsprozess beeinflussen. Diese genetischen Veränderungen könnten das Verhalten von Zellen in der Tumormikroumgebung von Hirnmetastasen, insbesondere im Vergleich zu extrakraniellen Tumoren, determinieren. Das Ziel dieser Forschungsarbeit war, die reziproke Interaktion zwischen Tumorzellen zerebraler Melanommetastasen und Astrozyten zu untersuchen. Der Fokus lag hier auf der Modulation zellulärer Funktionen. Daher wurde primär der Fokus auf die Analyse von Proliferationsaktivität, Migration und Invasionsverhalten gelegt.

Methodik: Um Melanomzellen mit unterschiedlichen gewebsspezifischen Charakteristika zu gewinnen und vergleichen zu können, wurden Melanom Zelllinien aus verschiedenen metastasierten Geweben von MT/*ret* Mäusen gewonnen. Nach in vitro Aktivierung von Astrozyten mit Melanomzellen konditioniertem Medium und Ko-Kulturen wurden Ki-67 Immunfluoreszenz Färbungen angefertigt, um den Einfluss auf die astrozytäre Proliferation zu analysieren. Veränderungen in der astrozytären Genexpression wurden mittels RT-qPCR analysiert. Um den Einfluss von Astrozyten auf die Migration und Invasion von Melanomzellen zu erforschen, wurden Migrations und Invasions Assays durchgeführt.

Ergebnisse: Unsere Analyse der reziproken Interaktion zwischen Melanomzellen und Astrozyten ergab, dass ein direkter Kontakt zwischen beiden Zellen erforderlich ist, um eine gesteigerte astrozytäre Proliferationsrate zu erreichen. Allerdings kann bereits das Sekretom von Melanomzellen eine Modulation der astrozytären Genexpression bewirken, insbesondere der inflammatorischen Marker. Durch diese Modulation können Melanomzellen eine tumorfördernde inflammatorische Tumormikroumgebung erzeugen. Außerdem konnten wir zeigen, dass Astrozyten eine erhöhte Migration und Invasion der Melanomzellen auslösen und somit die Bildung von Hirnmetastasen unterstützen können.

Zusammenfassung: Astrozyten spielen eine wichtige Rolle in der Entwicklung von Hirnmetastasen. In der Tumormikroumgebung resultiert der Kontakt zwischen Melanomzellen und Astrozyten in einer tumorfördernden Eigenschaft der Astrozyten. Die gezielte Hemmung dieser Prozesse durch spezifische Zielmoleküle, könnte die Entstehung von Hirnmetastasen reduzieren und somit das Therapieansprechen von Hirnmetastasen auf eine Immuntherapie verbessern.

Abstract (English):

Background: Malignant melanoma ranks as one of the most lethal kinds of skin cancers, and it carries a significant risk of spreading to other organs if it is not treated in its early stages. Patients diagnosed with stage IV disease have a subsequent occurrence of brain metastases in approximately 40% to 50% of cases. In the tumor microenvironment, melanoma cells are in complex interactions with resident cells, including astrocytes. When astrocytes come in contact with melanoma cells, they can have pro-metastatic effects by releasing a variety of molecules that affect the gene expression of surrounding cells and the immunological microenvironment. It is already known that brain metastatic melanoma cells exhibit important genetic modifications for migration, intra- and extravasation and differentiation. These genetic variations could potentially influence the behaviour of tumor cells during the metastatic process, particularly when comparing their behaviour within the brain compartment to that of extracranial tumor cells. The ambition of this thesis is to investigate the reciprocal interplay between melanoma cells and astrocytes by analyzing its influence on the cellular function of astrocytes, including proliferation, migration, and invasion.
Methods: To analyse the interplay between astrocytes and melanoma cells, astrocytes were activated in vitro with melanoma cells conditioned medium (MCM) or co-cultured with melanoma cells from the MT/*ret* mouse model. Then, immunofluorescent staining with Ki-67 was done to assess the astrocytic proliferation. Alterations in the gene expression of astrocytes were analysed by RT-qPCR. Furthermore, migration and invasion assays were executed to analyse if astrocytes have an impact on melanoma cell migration and invasion using *in vitro* xCelligence.

Results: Direct contact between astrocytes and melanoma cells leads to increased proliferation of astrocytes. However, the melanoma cell secretome modulates astrocytic gene expression, particularly inflammatory cytokine expression. Through this modulation, melanoma cells can induce astrocytes to establish a tumor-promoting inflammatory microenvironment. Conversely, we could show that astrocytes increase melanoma cell migration and invasion, thus promoting the emergence of melanoma brain metastases.

Discussion: Astrocytes significantly contribute to the emergence of brain metastases in melanoma. Within the context of the tumor's surroundings, melanoma cells can induce tumor-promoting properties in astrocytes. Finding potential therapeutic targets of astrocytic activity could possibly decrease the formation of brain metastasis and enhance therapy response to immunotherapy.

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List of Abbreviations

ACM	Astrocyte conditioned medium
AJCC	American Joint Committee on Cancer
ALDH1	Aldehyde dehydrogenase 1
ALDH1L1	ALDH1 family member L1
BBB	Blood-brain barrier
BM	Brain metastasis
BRAF	B-Raf protooncogene
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CM	Cutaneous melanoma
CNS	Central nervous system
CTLA4	Cytotoxic T-lymphocyte-associated antigen 4
Cx43	Connexin 43
CXCL10	C-X-C motif chemokine ligand 10
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
ET-1	Endothelin ligand 1
ETAR	Endothelin A receptor
ETBR	Endothelin B receptor
FDA	Food and drug administration
GFAP	Glial fibrillary acidic protein
GLT1	Astrocytic glutamate transporter 1 (GLT1)
GM-CSF	Granulocyte-macrophage colony stimulating factor
ICI	Immune checkpoint inhibitors
INF-γ	Interferon-gamma
JAK	Janus kinase
KIT	KIT protooncogene receptor tyrosin kinase
LDH	Lactate dehydrogenase
MAA	Metastases-associated astrocyte
MAPK	Mitogen-activated protein kinase

MCR1	Melanocortin receptor 1
MCM	Melanoma cells conditioned medium
MDSC	Myeloid-derived suppressor cell
ME	Melanoma
MET	Mesenchymal-to-epithelial transition
MMP2	Matrix metalloproteinase 2
MSH	Melanocyte stimulating hormone
$NAD(P)^+$	Nicotinamide adenine dinucleotide phosphate
NF1	Neurofibromin 1
NF-kB	Nuclear factor kappa B
NK	Natural killer
PA	Plasminogen activator
PD1	Programmed cell death protein 1
PD-L1	Programmed cell death ligand 1
PI3K	Phosphoinositol-3-kinase
PTEN	Phosphatase and tensin homolog
RA	Retinoic acid
RAR	Retinoic acid receptors
RT	Room temperature
RXR	Retinoid X receptors
SFM	Serum free medium
STAT	Signal transducer and activator of transcription proteins
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor-beta
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor-alpha
TP53	Tumor protein p53
UVR	Utraviolet radiation
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor

1. Introduction

1.1 Malignant melanoma

Melanoma is a very aggressive tumor that evolves from the uncontrolled growth of melanocytes. It accounts for approximately 70% of fatalities associated with skin cancer, making it the most fatal kind of skin cancer (1,2). The occurrence of malignant melanoma is on a constant rise, even though there has been a substantial enhancement in the outlook for individuals diagnosed with late stage (IV-metastatic) melanoma (3). During the last years, immunotherapy and targeted therapy were approved and have reformed the management of many cancers, but advance-staged melanoma in particular (4).

Melanocytes derive from the neural crest. They primarily reside in the epidermis, hair follicles and mucosa, but also along the meninges and in the choroid membrane (5). Cutaneous melanoma is the predominant type of malignant melanoma. When keratinocytes experience DNA damage due to UV light exposure, they generate melanocyte stimulating hormone (MSH). MSH subsequently attaches to its respective receptor, melanocortin receptor 1 (MCR1), on melanocytes, leading to the synthesis and release of melanin (6). Through dendritic processes of melanocytes, a connection to neighboring keratinocytes can be built, which enables the transfer of melanin containing melanosomes. This determines the skin color and therefore protects against further DNA damage from ultraviolet radiation (UVR) (7).

Malignant melanomas emerge from malignant transformation of melanocytes through UV-light, particularly UV-B light. Hence, exposure to sunlight is the foremost factor that increases the risk of developing malignant melanoma (5). Other risk factors are the amount of melanocytic nevi, a family history of the disease, genetic predisposition, and a previous history of a malignant melanoma, as around 5% of individuals who have had a previous malignant melanoma will go on to develop additional primary melanomas (8).

Malignant melanoma has the cancer with the highest mutational load as a consequence of DNA damage or DNA replication errors (9,10). As shown in figure 1, during the first step of melanoma formation, a melanocyte obtains an initiating driver mutation that results in melanocyte hyperplasia and the formation of a nevi (11). The most common somatic mutations resulting from UV-light induced DNA damage are genetic changes in the B-Raf protooncogene (BRAF), neurofibromin (NF1) and NRAS gene (12–15). These genes have a vital function in cell proliferation. Next, the expansion phase follows, in which some melanocytic nevi develop into intermediate lesions and eventually into melanoma *in situ*. In this context, mutations in specific genes such as phosphatase and tensin homolog (PTEN), KIT protooncogene receptor tyrosin kinase (KIT), tumor protein p53 (TP53), cyclin-dependent kinase inhibitor 2A (CDKN2A), and telomerase reverse transcriptase (TERT) have been identified as relevant factors. These genes are integral to fundamental cellular processes: PTEN and KIT are recognized for their significance in growth and metabolism, while TP53 is known for its role in preventing to apoptosis. Additionally, CDKN2A influences cell cycle regulation, and TERT is essential for the replicative process (11,16–19). In

melanoma, these genetic mutations typically result in the anomalous stimulation of the RAS/RAF/MAPK signaling pathway and the phosphoinositol-3-kinase (PI3K)/Akt cascade, which then results in increased cell proliferation and survival (20). The primary melanoma eventually gets into the invasive phase. It consequently becomes malignant melanoma and forms metastases to distant organs (11,17). Only around one third of melanomas arise from preexisting nevi. The remaining melanomas are thought to develop *de novo*. Nevertheless, they could also arise from clinically undetectable precursor lesions and may follow a comparable trajectory as detectable lesions (11,17,21,22).

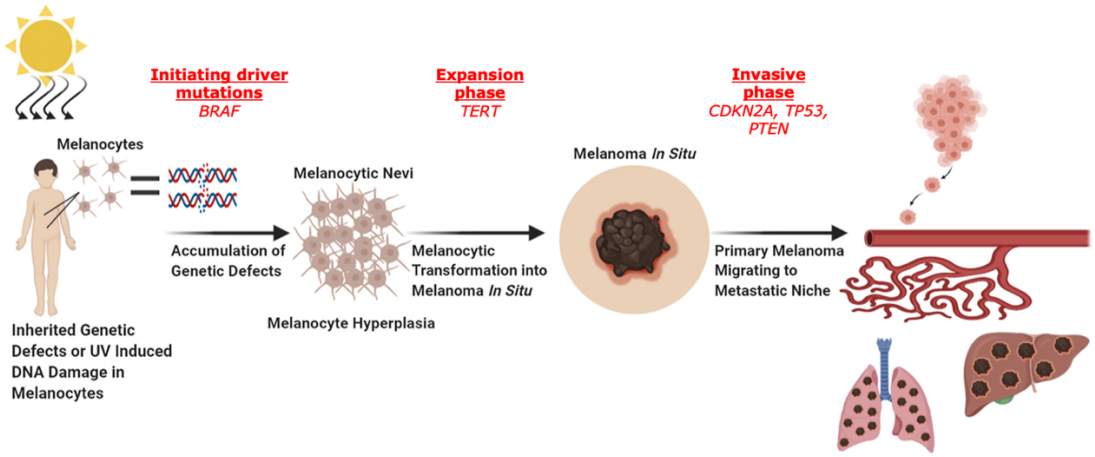


Figure 1: The development of malignant melanoma. First, a melanocyte obtains an initiating driver mutation which leads to the formation of a melanocytic nevi that is commonly marked by a BRAF mutation, followed by the expansion phase which describes the formation into a melanoma in situ with the acquisition of a TERT promotor mutation. Finally, the primary melanoma progresses into malignant melanoma and metastasizes into distant organs. Modified: (23).

For melanoma patients diagnosed with early-stage disease the - often curative - gold standard of care is surgical excision (24). Individuals diagnosed with metastatic melanoma have several available therapeutic options: Targeted therapy (BRAF and MEK inhibitors) can be used for BRAF-mutated melanomas, while immune checkpoint inhibitors (ICI) are available antibodies that target the surface protein cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) or programmed cell death protein 1 (PD1) (5). Further treatment options for metastatic disease are radiotherapy and chemotherapy.

Historically, systemic treatments could only achieve an average survival rate of around 9 months and an estimated 5-year survival rate of 10% (25). But since 2011, there were advances in immunotherapy which revolutionized treating metastatic melanoma. Overall, in individuals with American Joint Committee on Cancer (AJCC) stage IV melanoma, a five-year overall survival of approximately 50% could be approached (26). Pairing the anti-CTLA4 antibody ipilimumab with the anti-PD-1 checkpoint inhibitor nivolumab currently demonstrates the most favorable 5-year overall survival rate among all available treatment options. This treatment combination is also

notably effective in addressing melanoma brain metastases (4,26). Nevertheless, as resistance to targeted therapy develops rapidly, a considerable number of patients cannot be cured. Also, patients with primary resistance to immune checkpoint inhibitors or with difficulty treatable uveal or mucosal melanomas still are greatly in need of new treatment approaches to improve their prognosis (26). In spite of the current improvements in treatment, malignant melanoma remains a life-threatening disease and it is therefore fundamental to further investigate the pathophysiology of malignant melanoma and further research is needed (4).

1.2 Melanoma brain metastases

Metastasis is a multistage process which enables cancer cells to dissociate from the tumor of origin and spread to distant organs (27). During this process, melanoma cells obtain specific characteristics that allow enhanced proliferation, migration, invasion into adjacent tissues, intravasation into the blood stream or lymphatics and extravasation into the nearby tissue of distant organs (28).

As the brain has a lot of unique properties, such as a high energy and nutrient consumption, a highly controlled adaptive immunity and the selective blood-brain barrier (BBB), the brain is considered as an immune-suppressed organ (28). This immune privilege is under normal circumstances essential to prevent cytotoxicity, neurodegeneration and inflammatory brain swelling (29). However, Aspelund et al. and Louveau et al. have documented the presence of a functional lymphatic network adjacent to the dural sinuses. Furthermore, they have shown that antigens originating from the central nervous system (CNS) can trigger an immune reaction in the cervical lymph nodes (30,31). Therefore, immune reactions are not completely absent in the brain, but as an immune-privileged tissue, it still makes it necessary for metastatic cells to adapt to this unique environment to have the capability to form metastases.

The occurrence of brain metastases is fourfold more prevalent than of primary brain cancers. Therefore brain metastases are the leading source of malignancy in the CNS (32). Because of improved diagnostic imaging and better management of extracranial disease through systemic therapy, the incidence of brain metastases is steadily rising and affects approximately one third of cancer patients (33,34). After lung and breast cancer, which are the origin of brain metastases in 50% and 15% of the cases respectively, melanoma ranks as the third most prevalent source of brain metastases and accounts for approximately 10% (Figure 2) (35).

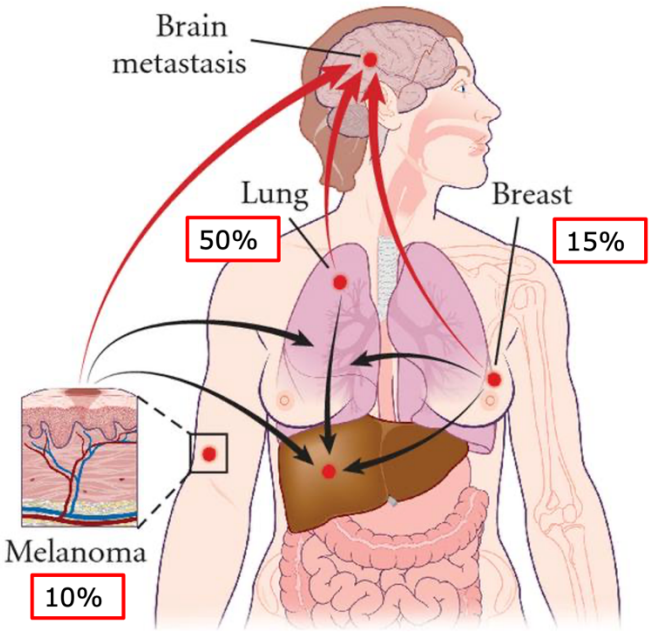


Figure 2: The diagrammatic process of metastasis. The formation of brain metastasis arises from spreading of cells from primary tumors via a circulatory system. The most frequent tumor origin of brain metastases are lung cancers in 50% of the cases, followed by breast cancers and malignant melanomas in 15% and 10% of the cases respectively (red arrows). Apart from brain metastases, malignant melanomas also frequently form metastases to other adjacent organs, for example the lymph nodes, lung, liver, and bones (black arrows). The inset shows how melanoma cells migrate form the primary tumor into the vasculature and afterwards disseminate to secondary organs. Modified: (35).

Melanoma brain metastases are linked to a notably grim outlook and result in mortality for 60-70% of affected patients (36). Relevant prognostic factors include the amount, extent and localization of brain metastases, the presence of extracranial metastasis, lactate dehydrogenase (LDH) level, patients age and tumor burden (36). As immunotherapy, targeted therapy and local therapy, like stereotactic radiosurgery and surgical techniques, have evolved, a collective improvement in the outcome of individuals with melanoma brain metastases to an average survival period of 1-2 years could be approached (37). Particularly patients with neurological symptoms, for example seizures or cognitive decline, are clinically challenging (36). Nevertheless, the overall brain is generally still an immune compromised tissue which makes the management of melanoma brain metastases particularly challenging (29). Consequently, it is essential to better understand the processes in the brain.

1.3 Migration and invasion of melanoma cells

Melanoma metastasis is predominantly driven by deviant cell motility. To date, there are only a limited amount of treatments accessible that target the specific inhibition of tumor cell motility, thus hindering the migration and infiltration of melanoma cells during the metastatic process (38). Throughout the metastatic process, melanoma cells can disseminate to distant organs through

intravasation through the vascular and lymphatic system. The type of migration, referred to “pericytic mimicry”, involves tumor cells moving independently of intravasation, where they meander along the external border of blood vessels (39,40). There have been reports that melanoma cells can exhibit “vasculogenic mimicry”. In the course of this procedure, the tumor is nourished by *de novo* formation of vascular networks, which promotes tumor perfusion (41,42).

A recurrent event in metastasis is epithelial to mesenchymal transition (EMT). EMT is a procedure that increases both cell adhesion and motility. This accelerates the escape of tumor cells from the place of origin (43,44). Compared to the cells of the tumor origin, metastatic cells are generally less differentiated. Therefore, the reverse mesenchymal to epithelial transition (MET) process is necessary to finish the advancement of the formation of metastatic tumors (45). Kienast *et al*. evaluated that the stages of the melanoma metastatic process involve cell arrest at junctions of blood vessels, initial extravasation, continual proximity to micro-vessels, and perivascular expansion through vessel co-option (46).

Cell motility is driven by the cytoskeleton, which consists of actin filaments, microtubules, intermediate filaments, and septins (47,48). During the cellular streaming, tumor cells can behave semi-collectively, for example they can infiltrate in a linear fashion, trailing a guiding cell (49). The degree of extracellular matrix (ECM) confinement represents another important feature that influences cell migration (50). For instance, constrictions display a mechanical challenge for cells to move through tight spaces. Studies have illustrated that the capability of cancer cells to contract, which is a crucial necessity for their migration, is restricted by the extent of nuclear compression. This nuclear limit of migration corresponds to just about 10% of the nuclear cross-section (51,52). When nuclear distortion surpasses this threshold, cells transition to migration modes that increase the breakdown of the ECM. One example of this is evident in the transition of amoeboid to mesenchymal migration. Alternatively, cells may undergo nuclear envelope rupture, which could potentially impact cell viability (52–55).

Within the brain, the motility of melanoma cells is controlled by the diameter of the vessels. This implies that during the spreading process, melanoma cells physiologically get stuck in the thin brain capillaries (46,56). It has been affirmed that various adhesion molecules are engaged in facilitating the attaching of metastatic cells to endothelial cells in the brain, but the exact mechanism remains unknown (28).

Every tumor shows a unique pattern of dissemination and preferential organ to form metastasis. For example colon cancer most commonly disseminates to the liver, while prostate cancer most frequently forms bone metastases (57). This organo-tropism can partly be explained by the system of venous drainage. Besides that, recent studies indicate that there are cellular and molecular programs, which guide tumor cells to specific organs (58). Metastatic melanoma cells most commonly exhibit a tropism towards the skin, liver, lung, small bowel or brain (59). How melanoma cells acquire this organ-specific tropism is yet unknown, but one proposed theory is that cytokines and chemokines have a pivotal function in the determination of this process (60,61). For example, research has indicated that the occurrence of the chemokine receptor CCR7 in murine melanoma

cells encourages melanoma to spread to lymph nodes and the brain, whereas CXCR4 enhances the tendency of melanoma to spread to the lungs (62,63). Furthermore, studies have demonstrated that the CXCL10-CXCR3 axis participates in attracting melanoma cells to the brain (1). This supports the theory that the metastasis of tumors to specific anatomical locations is influenced by the reciprocal interaction between tumor cells and does not happen randomly (58,64). As the majority of cancer-related deaths stem from metastatic conditions, it is imperative to do further research on tumor cell migration and invasion.

1.4 The brain tumor microenvironment

The tumor microenvironment (TME) confines a multifaceted and heterogenous system of cancer cells and non-cancerous cells and is a known critical regulator of cancer development and progression. The cellular compound of the TME also has a significant impact in metastatic malignancies. In the regard of brain metastasis, there are complex interactions between brain resident cells (65,66). The extracellular matrix consists of distinct cell types that reside in the tissue, such as microglial cells, astrocytes, and neurons. Also characteristically to the brain is the BBB, which distinguishes it from other tissues (29). Studies have shown that alterations in the TME advance clinically relevant brain metastasis and that cells actively participate in the advancement of cancer progression via interactions with tumor cells (1,67). The formation of such a complex TME among others, contributes to drug resistance. Therefore, to be able to design new TME-targeted interventions and consequently to improve overall therapy response, it is important to do further research about the unique properties of the brain.

During cancer progression, a variety of molecules play an important role, including cytokines, chemokines, and growth factors. Melanoma cells own the capability to shape their surrounding environment by releasing modulatory substances, which subsequently facilitate the progression of metastasis (68). In addition, they can secrete inhibitory factors aimed at preventing the identification and development of immune cells with an active role, among other signaling processes (69). Nevi and primary melanomas (< 1 mm thickness) exhibit minimal expression of tumor necrosis factor-α (TNF-α), transforming growth factor-beta (TGF-β), IL-8 and c-kit. However, melanomas in their advanced stages typically display elevated levels of TNF-α, TGF-β, IL-1, IL-8 and granulocyte-macrophage colony stimulating factor (GM-CSF) (70). Therefore, different mechanisms contribute to the melanoma cell's ability for rapid growth, invasion, and metastasis. This points out the significance of the TME and its part in the immune response throughout the progression of melanoma.

Several studies have revealed that the metastases formation in malignant melanoma is the result of genetic mutations. Research has demonstrated that the alteration of the TME happens to establish a favorable context for metastasis and is done by modifying the expression of tumor favorable proteins (5). For example, in the TME of brain metastasis, neurons are mostly seen as passive bystanders. However, microglia can activate T cells to trigger the adaptive immune response against tumor cells, and through the production of inflammatory-promoting molecules,

they can also induce the breakdown of the BBB (71–73). Astrocytes make up the greatest proportion of cells in the CNS and are involved in disease progression of multiple CNS malignancies. Nevertheless, the function of astrocytes in the development of melanoma brain metastases is not yet understood (74). Therefore, in this dissertation the focus was put on the inquiry of the reciprocal interplay of malignant melanoma cells and astrocytes.

1.4.1 Astrocytes

Astrocytes are glial cells which are exclusive to the CNS. They are the predominant cell population in the CNS. They have a crucial function in homeostasis, tissue repair and communication that is specific to the tissue, such as in the context of brain metastasis (1). Upon brain injury astrocytes become reactive. This process is called reactive astrogliosis, which is characterized by an astrocytic hypertrophy, glial fibrillary acidic protein (GFAP) upregulation, elevated synthesis and restructuring of proteins found in intermediate filaments and elevation in the release of inflammatory-promoting cytokines and chemokines (75). The stimulation of astrocytes and the onset of inflammatory reactions in the CNS can be triggered by microglia and immune cells that have been activated. This process is initiated in the course of brain metastasis formation (1,76). Interestingly, research has indicated that every cell type in the CNS can secrete factors that induce astrogliosis (77).

In the metastatic process, astrocytes were shown to initially prevent early stages of metastatic brain colonization by tumor cell killing, but later on, the tumor cell-astrocyte interaction results in tumor promoting properties of astrocytes (78–80). Beginning with the first interaction with metastatic cells, astrocytes secrete plasminogen activators (PA's) which causes the transformation of plasminogen into the protease plasmin, resulting in the killing of cancer cells that cross the BBB (79). However, some cancer cells can inhibit this anti-metastatic process by producing anti-PA serpins and therefore are able to continue with the colonization of the brain.

During the metastatic progression, astrocytes can acquire pro-tumorigenic features which in the case of malignant melanoma is believed to be caused by reprogramming of the astrocytes by the cancer cells. To give an example, tumor cells were shown to induce IL-23 secretion in astrocytes, resulting in an upregulation of tumor cell-derived matrix metalloproteinase 2 (MMP2), which then increases the infiltration capacity of brain-metastasizing melanoma cells *in vitro*. For instance, pharmacological inhibition of IL-23 can decrease melanoma invasion *in vitro* (81). Additionally, astrocytes were linked to promoting the advancement of tumor cells that spread to the brain through inflammatory-promoting signalling, but the underlying mechanisms are still unknown. Cancer cells that have spread to the brain hijack the initiation of inflammatory-promoting signalling in astrocytes to bolster their metastatic capabilities (1).

Another way of communication between melanoma cells and astrocytes is via extracellular vesicles. It is known that metastatic melanoma cells can secrete exosomes which play a main role in creating a favorable environment for metastasis (67). Those extracellular vesicles are capable of activating pro-inflammatory signaling in astrocytes and therefore reprogram tumor-promoting

functions (67). However, the exact mechanism underlying this tumor-promoting reprogramming remains unknown.

Moreover, through gap junctions, astrocytes can for instance communicate with surrounding neurons to ensure adequate activity of neurons, transmission of signals at synapses, providing energy support and regulating blood circulation (82). Interestingly, brain metastatic cells are known to be capable to form gap junctions with astrocytes, creating a physical connection that enables the transfer of signaling molecules between the cells (83–85). Gap junctions are formed by connexins. The most prevalent astrocytic connexins in the human brain are Cx43 (GJA1) and Cx30 (GJB6) (82). The direct contact through gap junctions can benefit the cancer cells through astrocyte-induced altered gene expression in cancer cell lines (Figure 3) (84). Klein *et al.* have displayed that astrocytes support the expansion of tumor cells that have spread to the brain through inflammatory-promoting signaling (81). Besides, evidence has indicated that the cancer cell generation of IL-6 and IL-8, relies on the formation of gap junctions with astrocytes (86). This IL-6 and IL-8 generation is accountable for the induction of the gene expression of the endothelin receptors, specifically the endothelin A receptor (ETAR) and endothelin B receptor (ETBR) on cancer cells and endothelin ligand 1 (ET-1) on astrocytes respectively and therefore influences both, tumor cells and astrocytes (86).

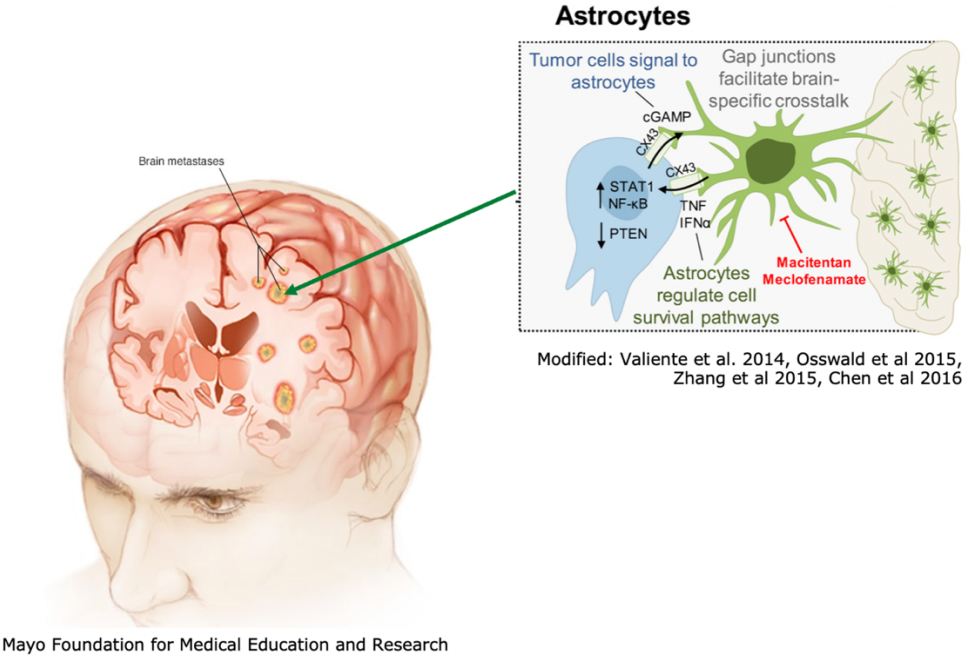


Figure 3: Astrocytes in the brain tumor microenvironment. Astrocytes establish functional gap junctions with cancer cells in brain metastases via the connexin Cx43. This enables the transfer of cGAMP and other signaling molecules which influences signaling pathways in each cell. The pharmaceuticals Macitentan (an antagonist of endothelin receptors) and meclofenamate (a cyclooxygenase inhibitor that regulates gap juctions) can inhibit this interaction. Modified: (29,87).

Only a few astrocyte-based therapies treating brain metastasis exist, including Macitentan and Gap junction inhibitors (figure 3). Macitentan is BBB penetrable inhibitor of the endothelin receptors A and B that has received approval from the FDA and primarily treats pulmonary arterial hypertension. However, it has also been shown that alongside paclitaxel, macitentan substantially enhances the survival rate in mice suffering from brain metastases originating from breast or lung cancer (88,89). Besides, inhibition of gap junctions by using the pan-Cx inhibitor carbenoloxone was able to increase cancer cell sensitivity to chemotherapy (85). Moreover, meclofenamate (a Cx43 gap junction inhibitor) and tonabersat (an inhibitor of astrocytic gap junction-mediated processes) which are also both FDA-approved, significantly decreased lung and breast cancer brain metastatic tumor burden (83). Until now, there are few effective treatment options for melanoma brain metastases, so it is essential to do further research about melanoma brain metastases to be able to discover new targeted therapy options to improve patients' diagnosis.

In glioblastoma multiforme, multiple signaling pathways between astrocytes and glioblastoma cells are already well understood (90). Glioblastoma cells activate astrocytes which results in reactive astrogliosis and furthermore enhances tumor progression, proliferation, and migration (90). Upon activation, astrocytes secrete inflammatory cytokines, chemokines, and other soluble substances, thereby inducing neuroinflammation (90). For example, astrocyte-glioma cell interaction results in enhanced expression of the GFAP and the connexin 43 (Cx43) (91). Research has indicated that in the brain TME, astrocytes exhibit specific gene signatures that are linked to glioblastoma that possess distinct genomic alterations (92,93). This suggests that astrocytes play a key role within the glioblastoma multiforme TME and therefore possibly also in melanoma brain metastasis.

1.4.1.1 Astrocytic gene expression

1.4.1.1.1 Glial fibrillary acidic protein (GFAP)

GFAP is one of the key astrocyte markers. Apart from vimentin, nestin, and synemin, it is a prominent intermediate filament protein in astrocytes. Its synthesis is induced in response to brain damage or neurodegeneration and is recognized to be upregulated in the mature human brain. There are about eight different isoforms of GFAP of which each may have a different function (94).

GFAP maintains the astrocyte mechanical strength and cell shape and participates in astrocyte functions such as tissue repair, synaptic plasticity, reactive gliosis, cell motility and migration (94). For example, Elobeid *et al.* illustrated that enhanced GFAP expression causes a reduction in the mobility of astrocytic cells (95). Furthermore, it has been demonstrated that through GFAP's involvement in mitosis and cell division, its increased expression results in decreased astrocytic proliferation, increased cell death and therefore reduced astrocyte growth (96–98). However, even though GFAP is prevalently used as an astrocyte marker, its exact functions remain unknown.

Upon CNS damage, reactive astrogliosis happens which is marked by an upregulation of GFAP expression as shown in figure 4 (99). Nawashiro et al. could show that mice who lack GFAP expression are more sensitive to cervical spinal cord injury, so GFAP actually has a significant role in protecting the brain against mechanical stress (100). Furthermore, they found that GFAP lacking mice are more susceptible to cerebral ischemia and neurotoxicity and that several days after cerebral ischemia, they showed neurodegeneration while mice with the normal, unaltered genetic configuration did not exhibit any evidence of degeneration of the CNS (101). Therefore, astrocytes with normal GFAP function are necessary for brain protection and may have a major function in the preservation of neurons.

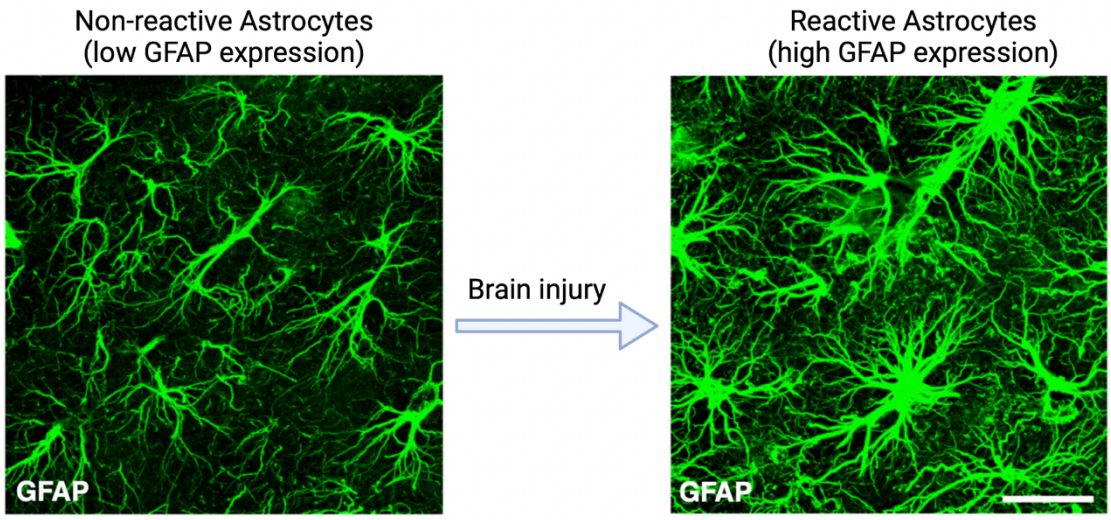


Figure 4: In Astrogliosis, reactive astrocytes are marked by an upward regulation of the intermediate filament protein called GFAP and hypertrophy of cellular processes. This can be visualized by GFAP immunofluorescence staining as shown in the right picture. Modified: (102).

Interestingly, among other diseases, Alzheimer's disease exhibits increased GFAP mRNA and protein levels. The enhanced GFAP expression is hereby normally considered secondary to neurodegeneration. Contrary, decreased GFAP has been proven to be linked to the rapid proliferation of gliomas and is more distinctive in high-grade gliomas in contradistinction to low-grade gliomas (94,103). Also, GFAP in the blood serum is an important diagnostic indicator for glioblastoma multiforme (104).

1.4.1.1.2 Aldehyde dehydrogenase 1 (ALDH1)

ALDH1 pertains to the detoxifying enzyme family and is primarily expressed in cortical and spinal cord astrocytes but is distributed all over adult organs (105). The three main isotypes of ALDH1 are ALDH1A1, ALDH1A2 and ALDH1A3. As with GFAP, ALDH1 expression is also upregulated upon reactive gliosis.

ALDH1 detoxifies endogenous and exogenous aldehyde substrates through nicotinamide adenine dinucleotide phosphate ($NAD(P)^+$)-dependent oxidation (106,107). During the metabolic processes involving amino acids, alcohols, lipids, and vitamins, endogenous aldehydes are generated. Exogenous aldehydes result from the metabolic process of a broad assortment of environmental factors, for example cigarette smoke, vehicle exhaust fumes and cytotoxic drugs (108). The ALDH1 family member L1 (ALDH1L1) is the folate enzyme 10-formyltetrahydrofolate dehydrogenase that transforms 10-formyltetrahydrofolate to tetrahydrofolate and therefore fulfills a crucial function in the synthesis of new nucleotides and the regeneration of methionine and therefore has a great impact on cell division and growth (109).

Cytosolic ALDH1 proteins catalyze the oxidation of retinal (retinal aldehyde) to retinoic acid (RA). RA attaches to nuclear RA receptors (RAR) and retinoid X receptors (RXR), performing a central role in the control of gene expression. For that reason it is important for regular cell growth, cell differentiation, cell development and the upkeep of organs (110).

Interestingly, high ALDH1 activity is linked to an unfavorable cancer prognosis and metastatic potential in several cancers, including malignant melanoma (110). Therefore, ALDH1 can serve as an indicator for drug resistance and disease progression in melanoma patients (111–113).

1.4.1.1.3 Astrocytic glutamate transporter 1 (GLT1)

Glutamate serves as the primary excitatory neurotransmitter within the CNS (114). GLT1 is the major astrocytic glutamate transporter. It is known to be upregulated in astrocytes during maturation and development, but in contrast to GFAP and ALDH1 it is downregulated during reactive gliosis (105,115). GLT1 is the primary transporter that takes up synaptic glutamate to keep an optimal extracellular glutamic level as elevated levels of extracellular glutamate are recognized to be connected with excitotoxic damage to neurons (116). There is increasing evidence that excitotoxicity is linked to a diversity of neurological conditions like Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (114,117,118). Also, GLT1 protein generation has been demonstrated to be significantly enhanced in glioblastoma cells (119).

Excitotoxicity is not limited to brain diseases such as epilepsy, stroke and neurodegenerative diseases, as it is also observed in brain tumors (120). During tumor cell invasion into surrounding brain tissue, neuron death happens due to excessive glutamate levels in synaptic clefts. Hereby, the increased ubiquitination-mediated degradation of GLT1 in brain tumors results in excessive amounts of glutamate in the synaptic cavity (121). Furthermore, glutamate is the major bioenergy substrate for brain cell and cancer cell growth (122). Additionally, it has been previously stated by Haowei Yi *et al.* that excessive extracellular glutamate can activate glutamate receptors on cancer cells resulting in malignant growth (122). Therefore, GLT1 has tumor promoting properties.

1.4.1.1.4 The role of astrocytes in inflammation: IL-1β, IL-17 and TNF- α

The secretion of cytokines by tumor cells or immune cells mediates chronic immune response and enhances the maturation and spread of the tumor (123). In the CNS, activated glial cells secrete multiple inflammatory mediators through which they form a chronic inflammatory reaction and therefore contribute to the innate immune response (124). Astrocytes secrete a diversity of those cytokines, embracing IL-1, IL-17 and TNF-α (125–128). As astrocytes represent the predominant type of glial cells, they hereby play a particularly crucial role (129).

IL-1β is primarily secreted by cells of the innate immune system and initiates signaling pathways that support cell survival and growth through its main signaling pathways, the NF-kB and MAPK signalling pathways. This results in the upregulated transcription of inflammatory genes, which generally promotes cancer progression. In melanoma, tumor-associated macrophages are the main source of IL-1β secretion. Formerly, it has been documented to be necessary for metastasis, invasiveness and angiogenesis throughout the induction of vascular endothelial growth factor (VEGF) and lymphotoxin, but conversely it has also been described to have some anti-tumorigenic effects (130–133). For instance, IL-1β promotes melanoma hepatic metastasis by increasing the synthesis of vascular cell adhesion molecule 1 (VCAM-1) and B16 melanoma mice treated with an IL-1 receptor antagonist demonstrated reduced tumor growth and lung metastasis (134,135). Therefore, the IL-1 signaling axis could be a prospective target for melanoma therapy. To mention some examples, IL-1 can influence tumor cells to produce VEGF for angiogenesis, as well as IL-6 and TNF for inflammation caused by the tumor. Additionally, IL-1 can stimulate the synthesis of several other cytokines, chemokines, and growth factors, thereby promoting the progression of cancer.

There are six different isotypes of IL-17, encompassing IL-17A through IL-17F (136). Within the tumor's surroundings, IL-17 is primarily produced by Th17, but is also generated by γδT cells, natural killer (NK) cells, neutrophils, and eosinophils (137). It is recognized to trigger the stimulation of astrocytes and the upregulation of VEGF through the JAK/STAT signaling pathway and therefore has significant impact in neuro-inflammation (138). It has furthermore been indicated that IL-17 works in synergy with IL-1β, IL-22, IFN- γ, TNF-α and other cytokines (139). Moreover, IL-17 is involved in pro-tumorigenic and anti-tumorigenic processes. Pro-tumorigenic effects of IL-17 such as enhanced proliferation, inhibition of apoptosis, immune evasion through the recruitment of myeloid-derived suppressor cells (MDSC's) and regulatory T cells and increased angiogenesis and metastasis were identified. On the contrary through the recruitment of cytotoxic and helper T cells and dendritic cells, enhancement of NK cell activity, generation and stimulation of cytotoxic T cells and the promotion of neutrophil infiltration, IL-17 creates an unfavorable inflammatory microenvironment for cancer cells and therefore also has its anti-tumorigenic effect (140). In a melanoma mouse model it has been demonstrated that IL-17 enhanced the expansion of melanoma cell lines, while silencing the IL-17 receptor antagonist reduced proliferation, migration and invasion, as well as, VEGF and matrix metalloproteinase production (141). Further studies showed reduced melanoma growth in mice with a IL-17 knockdown (142). While it is reported that

the IL-17 signaling axis has several antitumor activities, blood tests and tissue samples mainly indicate that elevated levels of IL-17 favor tumor growth and metastasis (143).

TNF-α is an abundant cytokine found in the tumor microenvironment with inflammatory and anti-inflammatory functions. It is mainly released by tumor-associated macrophages and induces chronic inflammation (144). TNF-α engages in proliferation, survival, epithelial-to-mesenchymal transition, and metastasis during cancer development. However, the function of TNF-α in tumor development remains controversial: it has a crucial role in apoptosis and necrosis which could prevent cancer development, but it also has its tumor promoting properties by being involved in the MAPK and NF-KB pathway. TNF-α was shown to increase the gathering and function of regulatory T cells, B cells and MDSC's, which negatively modulate the immune response (145). Furthermore, it can induce $CD8^+$ T cell death and hinder the infiltration of $CD8^+$ T cells (146–148). In melanoma, $CD4^+$ tumor-infiltrating lymphocytes also produce TNF-α which leads to an inhibition of $CD8^+$ T cell response and it was also shown that TNF-α triggers melanoma dedifferentiation as well as it promotes immune escape and relapse (149). Also, it can elevate the synthesis of programmed cell death ligand 1 (PD-L1) and therefore restrain the immune response (150). Interestingly, Bertrand *et al.* could show that 1 in experimental melanoma, overcoming resistance to anti-PD-1 can be achieved by blocking TNF-α (145).

1.4.1.1.5 C-X-C motif chemokine ligand 10 (CXCL10)

In addition to their role in neuroinflammation, astrocytes also participate in attracting cancer cells to the brain (1). The course of attracting melanoma cells to the brain has been described to be supported by the CXCL10-CXCR3 signaling axis (Figure 5) (1). CXCL10 is part of the CXC chemokine family and serves as the ligand for its corresponding receptor CXCR3. CXCL10 is produced and released by an assortment of cells, such as monocytes, endothelial cells, fibroblasts and astrocytes in reaction to interferon-γ (INF-γ) and functions as a modulator of the migration of macrophages, monocytes, NK cells and T cells (151). It is recognized to be elevated not only during the course of reactive gliosis but also within the microenvironment of brain metastases and interestingly, high CXCL10 levels correlate with a bad clinical response in advanced melanoma patients (152,153). Moreover, Doron et al. could show that CXCL10 expression is increased in astrocytes associated with metastasis and that its release is necessary for astrocyte-induced attraction of melanoma cells to the brain (1).

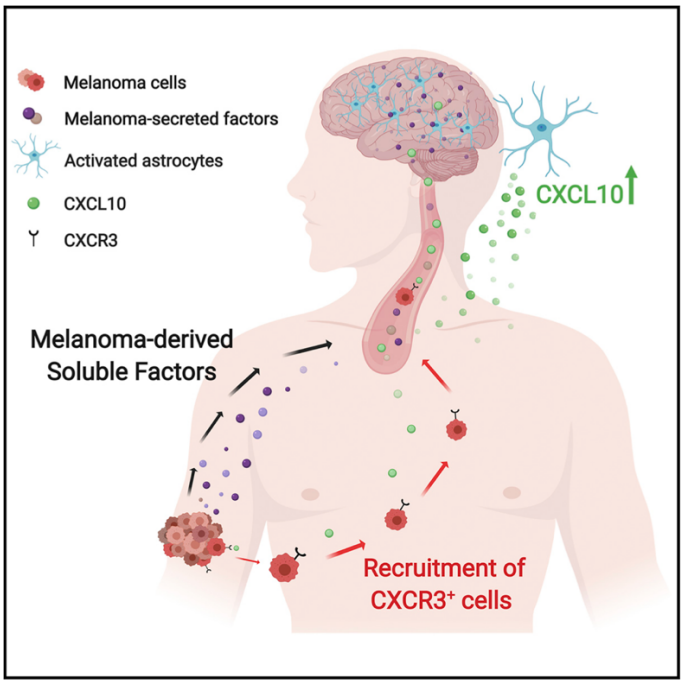


Figure 5: Astrogliosis induces melanoma's tendency to metastasize to the brain through the CXCL10-CXCR3 signaling axis. CXCL10 is increased in astrocytes connected with metastases and enhances melanoma cell migration towards astrocytes by binding to its corresponding receptor CXCR3. CXCR3 is known to be increased in brain-tropic melanoma cells. (1)

After the spontaneous formation of brain micro-metastases, astrocytes already exhibit increased CXCL10 expression in the tumor microenvironment. CXCL10 is also present in astrocytes associated with macro-metastases, implying that CXCL10 is involved in various steps of the formation of metastases. Furthermore, brain-tropic melanoma cells show an enhanced presence of the CXCL10 receptor, CXCR3. Studies have demonstrated that when CXCR3 expression is targeted, it results in the inhibition of melanoma brain metastasis (1). On the contrary, it has also been shown, that astrocyte secreted CXCL10 mobilizes T cells. As a great density of T cells in the brain is linked to a better life expectancy in patients with brain metastasis and as CXCR3 expression on cytotoxic T cells is connected with a more favorable reaction to anti-PD-1 treatment in a melanoma mouse model, it is critical that future therapeutic strategies aimed at the CXCL10-CXCR3 axis needs to be addressed carefully (154,155).

1.4.1.1.6 Connexin 43 (Cx43)

Gap junctions are composed of two opposing hemichannels. Those hemichannels are known as connexons, and each connexon is made up of six connexin subunits. Cx43 gap junctions are modulators of important immunological processes, including anti-tumor responses. As previously mentioned, Cx43 is the predominant connexin in astrocytes and participates in the direct communication between astrocytes and cancer cells. Once gap junctions are formed, cells that metastasized to the brain have the capability to transmit the second messenger cGAMP to astrocytes.

The following stimulation of the STING signalling pathway and the generation of inflammatory markers, including INF- α and TNF which trigger the stimulation of the STAT1 and NF-kB signalling pathways within cells that metastasized to the brain, further increase cancer progression and chemoresistance (83). For example, co-culturing tumor cells with astrocytes has been demonstrated to provide protection to tumor cells against chemotherapy (83). Aforementioned, pharmacological blocking of these gap junctions with meclofenamate and tonabersat suppresses brain metastasis in mice (83). Furthermore, Cx43 has also been described to be linked to decreased survival without metastasis in individuals with non-small cell lung cancer (NSCLC). These insights demonstrate the pertinence of Cx43 in brain metastasis.

2. Material

2.1.1 Laboratory Equipment

Material/Device	**Company**
Allegra 25R centrifuge with microplate adapters	Beckman Coulter
Automated Cell Counter	BIO-RAD
Bench	Nuaire
Cell culture dishes Cellstar® (100mm)	Greiner Bio-One GmbH
Cell culture flasks Cellstar® (25 cm^2, 75 cm^2)	Greiner Bio-One GmbH
Cell culture plates Cellstar® (6 well, 12 well, 24 well, 96 well)	Greiner Bio-One GmbH
Centrifuge	Hettich
Counting slides	BIO-RAD
EVOS M7000	Invitrogen by Thermo Fisher Scientific
Falcon Tubes (15 mL, 50 mL) Cellstar®	Greiner Bio-One GmbH
Glass discs	
Incubator	Heracell
Microscope	Carl Zeiss
Microscope slides 76x26 mm	Engelbrecht Medizin-und Labortechnik GmbH
NanoDrop spectrophotometer ND-1000	NanoDrop Technologies
Optical adhesive cover	Applied Biosystems
Phase lock gel (2ml)	Eppendorf
RCTA CIM-plate 16	Agilent Technologies
Stainless steel mortar and pestle sets	Fisher
T3 Thermoblock	Biometra
Scale ABT120-5DNM	Kern & Sohn
Superfrost™ Microscope Slides	Epredia
Water bath	Gesellschaft für Labortechnik (GFL)

xCELLigence RTCA System	Roche
7900 HT real-time PCR instrument	Applied Biosystems
96-well real time PCR plate	Applied Biosystems

2.1.2 Chemicals

Name	**Company**	**Order number**
Agarose universal 500g	VWR Life Scientific	35-1020
Astrocyte conditioned medium	Provito	SC-M1800-57
Astrocyte Growth Supplement (AGS)	ScienCell	1853
DAPI	Sigma-Aldrich	D9542
DEPC	Carl Roth	K028.1
DMSO	Carl Roth	4720.3
dNTP set, 100 nM	Fermentas	R0141, R0151, R0161, R0171
Donkey Serum	Millipore	S30-100ml
Dream Taq Buffer (10X)	Thermo Fisher Scientific	B65
Dream Taq DNA Polymerase (5 U/µl)	Thermo Fisher Scientific	EP0703
DMEM	Gibco	41965-039
DPBS	Gibco	14190-094
EDTA	Sigma-Aldrich	E9884-100G
Ethanol absolute	Fisher Chemicals	12438760
Ethanol	Merck	1.00974.2511
Fetal Bovine Serum (FBS)	PAA	A15-101
Fluoromont-G	Invitrogen	00-4958-02
Formaldehyde solution	Sigma-Aldrich	F1635-500ML
Gelred	Biotium	41003-1
Glacial acetic acid	Merck	1.00063.1000
L-Glutamin (200 mM)	Gibco	25030-024

Matrigel	Corning	DLW356231
MOPS	Sigma-Aldrich	M3183-100h
PBS	Gibco	14190-094
Penicillin/Streptomycin	Gibco	15140-122
PFA	Riedel-de Haën	16005
Poly-L-Lysin (0,01% solution)	Bio-Sell	535686
PowerTrack SYBR green PCR master mix	Applied Biosystems	A46112
Random primers	Invitrogen	48190011
RNase-free DNase I (1 U/µl)	Thermo Scientific	EN0521
Roti-Load buffer	Carl Roth	T848.1
Sodium acetate	Sigma-Aldrich	S2889-250G
SuperScript™ II Reverse Transcriptase (200 U/µl)	Invitrogen	18064071
TNS	ScienCell	0113
Tris base	Millipore	648310-500GM
Triton ™ X-100	Sigma-Aldrich	X100-100ML
Trizol reagent	Invitrogen	15596026
Trypan Blue Solution	Sigma-Aldrich	T8154-20ML
Trypsin/EDTA (0,05%/0,02%) in PBS	Bio-Sell	501211
Tween® 20	Carl Roth	9127.1
100bp ladder	ThermoScientific	SM0322
2-Mercaptoethanol	Sigma-Aldrich	M3148-25ML

2.1.3 Cells

Name	Company	Order number
Astrocytes M-1800-57	Provito	SC-M1800-57
CM parental	Isolated by the working group from Prof. Dr. Iris Helfrich.	
BM-ME5		
BM-ME-M1731		
BM-ME-M2088		

2.1.4 Primers

PCR				
For	**Gene**	**forward primer (5'→3')**	**reverse primer (5'→3')**	**Reference**
Mus	M18S RNA	CGGCTACCACATCCAAGGAA	GCTGGAATTAC-CGCGGCT	Chand 2009
qPCR				
For	**Gene**	**forward primer (5'→3')**	**reverse primer (5'→3')**	**Reference**
Mus	ALDH1L1	GGCTCTTCACCTGGCATCTTA	GGCCCATAACCAGG-GACAAT	Cibelli 2021
Mus	GLT1	TGGTCACCATGCTCCTCATT	CAAAAGAATCGCCCAC-CACA	Cibelli 2021
Mus	GFAP	ACCTGCAGGAGTACCAG-GATCTAC	TCTGTACAGGAATGGT-GATGCG	Doron 2019
Mus/Rat	Cx43	CCTCCTGGGTACAAGCTGGT	CGATTTT-GCTCTGCGCTGTA	Cibelli 2021
Mus	Cxcl10	CACCATGAACCCAAGTGCTG	TTGCGAGAGG-GATCCCTTG	Doron 2019
Mus	IL-17	CTCAGACTACCTCAACCGTTC	TGAGCTTCCCAGATCA-CAGAG	Shan 2017
Mus	IL-1β	GCACTACAGGCTCCGAGAT-GAAC	TTGTCGTTGCTTGGTT-CTCCTTGT	Ajoy 2021
Mus	TNF-a	GGAACTGGCAGAAGAGGCACTC	GCAGGAAT-GAGAAGAGGCTGAGAC	Ajoy 2021
Mus	Hes-1	CTAT CATG-GAGAAGAGGCGAAG	CCGGGAG CTATCTTT-CTTAAGTG	Novaki 2021

Mus	Hes-5	CCAAGGAGAAA AACCGACTG	TCCAGGAT-GTCGGCCTTC TC	Novaki 2021
Mus/Rat	βActin	CGTTGACATCCGTAAAGACC	TCTCCTT-CTGCATCCTGTCA	Cibelli 2021

2.1.5 Kits

Name	**Company**	**Order number**
Revert Aid Minus First Strand cDNA Synthesis Kit	Thermo Fisher Scientific	K1632
RNA mini kit	Qiagen	74106
RNase-Free DNase Set	Qiagen	79254

2.1.6 Antibodies

Name	**Company**	**Order number**
Alexa Fluor 594, red-fluores-cent dye (goat anti-rabbit IgG)	Invitrogen	A11012
Ki-67 (rabbit anti-mouse IgG)	Abcam	ab15580

2.1.7 Software

Software	**Company**	**Link**
ImageJ 1.48x	NIH	https://imagej.nih.gov//jj
GraphPad Prism version 7.0 for Mac	GraphPad Software, La Jolla California USA	http://www.graphpad.com

2.1.8 Buffers and Solutions

50X TAE buffer	
Tris base	242g
Glacial acetic acid	57,1ml
EDTA (0,5 M)	100ml
10X MOPS buffer	
MOPS	42g
Sodium acetate	4,1g
EDTA (0,5 M)	20ml
DEPC (diethylpyrocarbonate)-treated water in pH 7,0	1l

2.2 Methods

2.2.1 Cell Culture

All melanoma cell lines originated from the spontaneous mouse model (Mt/ret) (156). The CM cell line derived from cutaneous melanoma cells and the BM-ME-M2088, BM-ME-M1731 and BM-ME5 cell lines originate from melanoma cells that metastasized to the brain. The CM parental cell line was established from cutaneous melanoma of the Mt/ret model. All tumor cell lines were established by the research group of Prof. Helfrich.

The melanoma cell lines (BM-ME-M2088, BM-ME-M1731, BM-ME5p, CM parental) were seeded in T75 cell culture flasks (T75 = 75 cm^2 grow surface) and cultivated in medium (DMEM) enhanced with 10% fetal calf serum (FCS), 1% penicillin/streptomycin (PS) and 1% glutamine. The melanoma cells were passaged twice a week into new cell culture flasks. Astrocytes, isolated from a postnatal day two C57BL/6 mouse brain, were planted in 6-well plates and cultivated in astrocyte medium enhanced with 2% fetal calf serum (FCS), 1% penicillin/streptomycin (PS) and 1% astrocyte growth supplement (AGS). When the astrocytes reached a 90% confluence, they were converted to a new 6-well plate. All cells were kept at 37°C in an atmosphere holding 5% CO_2.

2.2.2 Passaging of Cells

The tumor cells were transferred into a new flask when they reached a confluency of 75-85%. The solution was aspirated, the cells were rinsed with PBS and afterwards kept in trypsin/EDTA-PBS-solution at 37°C for 1-2 minutes, the cells became dislodged from the substrate. The trypsinized cells were then rinsed with complete-medium and the suspension was then centrifuged (25°C, 1000 rpm, 5 min.). The supernatant was detached, and the left-over cell pellet was redissolved in 1ml of fresh medium and splitted as listed below (Table 1).

The astrocytes were passaged when they reached a confluency of 80-90%. Subculturing of astrocytes was done by washing the astrocytes with PBS and then incubating them in a T/E and PBS solution for 1-2 minutes. Afterwards this solution was transferred into a tube with FBS. The flask was incubated for another 1-2 minutes (without solution) and then the residual astrocytes were collected by washing the flask twice with TNS. The tube was then centrifuged, and subsequently, the cell pellet was redissolved in astrocyte medium and afterwards moved to a PLL-coated flask.

Table 1: Tumor cell line splitting rate. Splitting rate of each tumor cell line and the corresponding flask and medium that were used.

Cell Type	**Splitting Rate**	**Flask**	**Medium**
BM-ME-2088	1:5	T75	DMEM
BM-ME-M1731	1:5	T75	DMEM
BM-ME5	1:5	T75	DMEM
CM parental	1:10	T75	DMEM
BM-ME5 fluc mCherry	1:5	T75	DMEM
CM-tyr-KO43 fluc mCherry	1:10	T75	DMEM

2.2.3 Coating plates with Poly-L-lysine (PLL)

To prepare poly-L-lysine (PLL) coated plates, 15µl of PLL-solution was put into 10ml of sterile water. The solution was then put into the wells and the plates were kept for a minimum of 60 minutes in the incubator (37°C and 5% CO_2). The plates were then used directly or kept in a - 4°C fridge until needed.

2.2.4 Melanoma cells conditioned medium preparation

To prepare MCM, 2×10^5 melanoma cells were placed in 10cm plates and cultured for 48 hours in a serum-free medium (SFM). To make sure that only the conditioned medium and no melanoma cells were collected, the sucked MCM was centrifuged (25°C, 1000 rpm, 5 minutes) and the supernatant was then collected. The MCM was always used fresh for the performance of experiments.

2.2.5 In vitro astrocyte activation by melanoma cells conditioned medium

2×10^4 astrocytes were placed in each well of PLL-coated 24-well plates with 10mm discs and incubated in astrocyte-conditioned medium (ACM) until they reached a confluency of 90%. Subsequently, the astrocytes were incubated for 24 hours with MCM or SFM as a control. Afterwards, the cells were treated with cold 4% paraformaldehyde (PFA) for 20 minutes at room temperature.

2.2.6 Coculture of astrocytes with melanoma

5×10^3 astrocytes were seeded in PLL-coated 4-ring dishes and incubated in ACM until they reached a confluency of 90%. The astrocytes were then cocultured with 2×10^3 melanoma cells for 24 hours.

2.2.7 Immunofluorescent cell staining

Following the 20-minute fixation of the astrocytes with 4% PFA and their subsequent washing with PBS, the cells were further rinsed threefold for 5 minutes with 1 × PBS/0,1% Tween at RT and then once for 5 minutes with 0,2% Triton in PBS. The cells were then blocked with 10% donkey serum in PBS for 30 minutes at room temperature. Subsequently, they were incubated overnight with the primary antibody (Ki-67), as listed below (Table 2). Antibodies were always dissolved in blocking solution. A control without primary antibodies was kept.

Table 2: Dilution rate of the primary antibody.

Primary antibody	**Dilution**
Ki-67	1:250

After the incubation of the cells with the primary antibodies, the cells were washed with PBS and afterwards subjected into incubation with the secondary antibody (AF594) and DAPI (blue, 1:1000 dilution) for 30 minutes at room temperature. The antibodies were dissolved in blocking solution. The incubation was always done in darkness.

Table 3: Dilution rate of the secondary antibody.

Secondary Antibody	**Color**	**Dilution**
Alexa Fluor 594	Red	1:500

Afterwards, the samples were rinsed with PBS and embedded in Fluoromont. The samples were left at 4°C to dry prior to microscoping.

2.2.8 Quantitative qPCR

2.2.8.1 RNA extraction

The RNeasy Mini Kit (Qiagen) was used for RNA extraction. The cellular pellet was resuspended in 350µl of buffer RLT (RNeasy Lysis Buffer, Qiagen) that contained 1% β-mercaptoethanol (β-Merc, Sigma). The cells were collected with the rubber policeman, put in an 1,5ml Eppi and vortexed. The lysate extract was then pippeted into a QIAshredder spin column (Qiagen) and subsequently transferred into a 2ml collection tube, after which centrifugation was performed for 2 minutes at 8000×g. Subsequently, 350µl of 70% ethanol was introduced to the homogenized lysate. The resulting suspension (700µl) was loaded into the RNeasy spin column, which was then positioned within a 2ml collection tube and subjected to centrifugation for 30 seconds at 8000 × g. For the initial washing step, 350µl Buffer RW1 was introduced to the RNeasy spin column and centrifuged for 30 seconds at 8000 × g. Next, a blend consisting of 10µl of DNase stock solution and 70µl of RDD buffer was included and enabled to incubate for 15 minutes at room temperature. For the second washing step 700µl RW1 buffer was introduced, and the solution was centrifuged for 30 seconds at 8000 × g. Following that, 500µl RPE buffer was introduced and centrifuged for 30 seconds at 8000 × g. Next, another 500µl of RPE buffer was introduced, and this time, the centrifugation continued for 2 minutes at 8000 × g. After this, dry centrifugation was performed for 1 minute at the highest pace. For the elution step, the columns were inserted into 1,5ml Eppendorf tubes, and 30µl of RNase-free water was included. Subsequently they were centrifuged for 1 minute at 8000 × g to collect the eluted RNA.

The quantification of isolated mRNA was performed employing spectrophotometrie (optical density at 280nm ad 260nm) with the NanoDrop spectrophotometer ND-1000. The RNA was preserved at -80°C. Further work was always done on ice.

2.2.8.2 RNA quality analysis

The RNA quality assessment was conducted through electrophoresis. Initially, 0,5µg of RNA was resuspended in 5µl RNAse-free water and combined with 15µl of Roti-Load Buffer. The suspension was then left to incubate for 15 minutes at 65 °C. Subsequently, electrophoresis of the samples was carried out in a 1X MOPS gel (as specified in Table 4) using running buffer (1X MOPS buffer) for a duration of 90 minutes at 100 V.

Table 4: 1X MOPS Gel.

Reagent	**Volume**
Agarose	2 g
Distilled Water	170 ml
Gel Red	8 µl
Formaldehyde solution (37,5%)	10.8 ml
MOPS buffer 10X	20 ml

The integrity of the total RNA was examined by observing distinct 28S and 18S RNA bands. To rule out any potential genomic DNA (gDNA) impurities in the RNA samples, a PCR test targeting the m18S RNA was conducted (Table 5). 1,5% agarose gel (Table 6) was used to visualize the genetic material.

Table 5: Reagents for m18S RNA PCR test.

Reagent	**Stock concentration**	**Volume 1x**
H_2O (Braun)	-	19,8 µl
dNTPS (Thermo Fisher Scientific)	10 mM	0,5 µl
Dream Taq Buffer (Thermo Fisher Scientific)	10x	2,5 µl
Dream Taq DNA Polymerase (Thermo Fisher Scientific)	5 U/µl	0,2 µl
m18S RNA forward	100 pmol/µl	0,5 µl
m18S RNA reverse	100 pmol/µl	0,5 µl
RNA	0,5 µg	1 µl

Table 6: 1,5% Agarose Gel.

Reagent	**Volume**
Agarose	1,5 g
TAE	100 ml
Gel Red	4 µl

2.2.8.3 Complementary DNA (cDNA) synthesis

The synthesis of cDNA was conducted by employing the Revert Aid Minus First Strand cDNA Synthesis Kit from Thermo Fisher Scientific. To generate cDNA, 500ng of RNA was combined with 1µl of random hexamer primers and 12µl of DEPC water. This mixture was thoroughly vortexed, followed by centrifugation and then left to incubate for 5 minutes at 65°C. Subsequently, 7µl of the master mix (detailed in Table 7) was introduced into each sample. After another round of vortexing, centrifugation, and a 5-minute incubation at 25°C, 1µl Revert Aid H Minus M-MuLV Reverse Transcriptase (200 U/µl) was introduced and the combination underwent incubation for 60 minutes at 42°C. Finally, it underwent an additional 5-minute incubation at 70°C to complete the cDNA synthesis process.

The cDNA quality was tested by the m18S RNA PCR test, using the identical procedure as earlier detailed in the RNA quality analysis. This involved using 1µl of cDNA. The cDNA samples were subsequently preserved at -20°C.

Table 7: Master mix reagents for cDNA synthesis.

Reagent	**Stock concentration**	**Volume 1x**
Reaction buffer	5x	4 µl
RiboLockRNase Inhibitor	20 U/µl	1 µl
dNTP Mix	10 mM	2 µl

2.2.8.4 Real Time-qPCR

The primer and probe sequences employed in the real time (RT)-qPCR are detailed in the Materials section. RT-PCR reagents (Applied Biosystems, Foster City, CA, USA) were used as set by the manufacturer's guidelines and outlined in Table 8. For the RT-qPCR, 1µl of the cDNA samples was combined with the RT-PCR reagents and the respective gene primers (refer to Table 10). The PCR process entailed the following steps: initial incubation at 50°C for 30 minutes, then denaturation at 94°C for 3 minutes, followed by repeating 24 cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 30 seconds, and concluding with a last extension at 72°C for 5 minutes. No ROX SYBR (Takyon) was used as a passive reference dye. The RT-qPCR was carried out utilizing the Applied Biosystems QuantStudio™ 1 Real-Time PCR System, and the cycle threshold (Ct) value for the astrocyte target gene was determined. The experiments were conducted in triplicates.

Table 8: Reagents for RT-qPCR.

Reagents	**Preparation for 1 sample (µl)**	**Preparation for 5,5 samples (µl)**
dH_2O	19,8	108,9
10X Dream Taq Buffer + 20 mM $MgCl_2$	2,5	13,75
dNTP'S 10mM	0,5	2,75
Primer forward 10 pmol/µl	0,5	2,75
Primer reverse 10 pmol/µl	0,5	2,75
Dream Taq DNA Polymerase 5u/µl, 500u	0,2	1,1
cDNA per well	1	

2.2.9 Real time cell analysis

2.2.9.1 In vitro activation of melanoma cells by astrocyte-conditioned medium (ACM) for migration assay

To prepare astrocyte-conditioned medium (ACM), astrocytes which reached a confluency of 80% in a 6-well-plate, were cultivated for 24 hours in astrocyte medium. To make sure that only the secretome of the astrocytes was then collected, the removed medium was centrifuged (25°C, 1000 rpm, 5 minutes) and then the supernatant was collected. Melanoma cells were then cultured for 24 hours in ACM before used for the migration assay.

2.2.9.2 Migration assay

As indicated in table 11, a certain quantity of melanoma cells was placed in 6-wells and cultured in DMEM 48 hours prior to the assay. After 24 hours, melanoma cells were incubated with ACM, which was generated as described above. To investigate, if the priming of the melanoma cells with ACM influences the migration of the tumor cells towards the astrocytes, a control group with melanoma cells, which were only incubated with DMEM was done.

For the migration assay, RTCA CIM-plates were used. In the lower chamber of the 16-well RTCA CIM-plates, either 2×10^4 astrocytes in 160µl astrocyte medium or 160µl DMEM as a control were seeded. In the top chamber, 6×10^4 cells of each melanoma cell line, which were previously incubated with ACM (primed) or DMEM only (unprimed) were seeded in 100µl DMEM (graph 1). The migration of the melanoma cells towards the astrocytes was then evaluated using the xCELLigence system at 37°C for 48 hours.

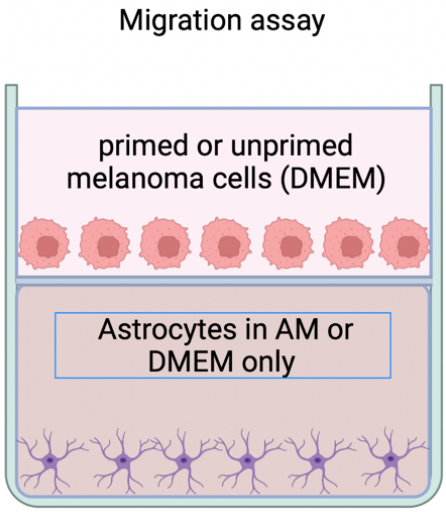


Figure 6: Schematic seeding of melanoma cells and astrocytes for the migration assay. In the upper chamber 6 × 10^4 cells of each melanoma cell line were placed in 100µl DMEM, which were either primed (incubated with ACM) or, as a control, unprimed (incubated with DMEM). In the lower chamber, either 2× 10^4 astrocytes in 160µl astrocyte medium were seeded or DMEM only as a control was used. Figure was created with BioRender.

2.2.9.3 Invasion assay

As indicated in table 11, a certain quantity of melanoma cells was put in 6-well plates and cultured in DMEM 48 hours preliminary to the assay. After 24 hours, the priming of melanoma cells with astrocyte conditioned medium followed as described above. The invasion assay was conducted with RTCA CIM-plates. The top chamber of the CIM-plates was covered with a diluted Matrigel (BD Bioscience) at a 1:60 ratio in DMEM and then was incubated for 4 hours before the melanoma cells were seeded. As already described in 6.2, in the lower chamber of the 16-well RTCA CIM-plates, either 2× 10^4 astrocytes in 160µl astrocyte medium were seeded or DMEM only as a control was used. In the upper chamber, 6 × 10^4 of the with ACM primed melanoma cell line were seeded in 100µl DMEM. The invasion of the melanoma cells towards the astrocytes was then evaluated using the xCELLigence system at 37°C for 48 hours.

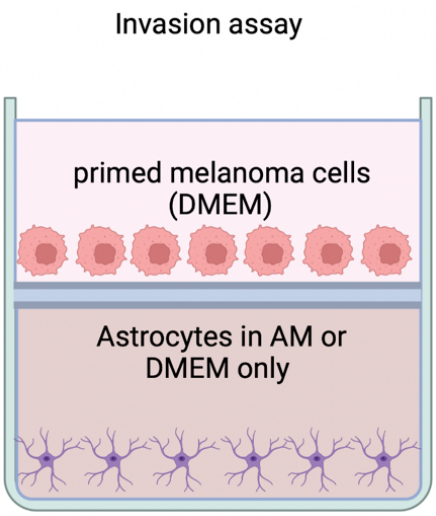


Figure 7: Schematic seeding of melanoma cells and astrocytes for the invasion assay. In the upper chamber 6 × 10^4 cells of primed melanoma cell line were placed in 100µl DMEM. In the lower chamber, either 2× 10^4 astrocytes in 160µl astrocyte medium were seeded or DMEM only as a control was used. Figure was created with BioRender.

Table 9: Cell number of melanoma cells for the migration and invasion assay. The cell count placed in 6-well plates 48 hours before the assay and the quantity of cells placed in 16-well RTCA CIM-plates on the day of the migration assay respectively.

Cell line	**Step1: Number of cells seeded in 6-well-plate**	**Step 2: Number of cells seeded in RTCA CIM-plate**
CM parental	1.1 × 10^5	6 × 10^4
BM-ME5	2.5 × 10^5	6 × 10^4
BM-ME-M2088	2 × 10^5	6 × 10^4
BM-ME-M1731	2 × 10^5	6 × 10^4

2.2.10 Statistical analysis

To compare to sets of entities using the mean values, the t-test was employed as the parametric method, while the Mann-Whitney-U-test was used for the non-parametric data. The p-values were calculated for both sides (two tailed). A significance threshold of $p \leq 0{,}05$ was deemed statistically significant. The analysis was executed using GraphPad Prism 8 software.

3. Results

3.1 The secretome of melanoma cells does not influence the proliferation of astrocytes

To analyze the reciprocal interplay between melanoma cells and astrocytes, we first had a look at the indirect influence of melanoma cells on astrocytes. We wanted to see if the secretome of melanoma cells influences the cellular function of astrocytes. For that, we did *in vitro* activation of astrocytes with melanoma cells conditioned medium (MCM). Each experiment was repeated three times to validate its reliability.

First, to see if the melanoma cell secretome influences astrocytic proliferation per se, we compared activated astrocytes with the astrocyte control group, which was solely incubated with serum free medium (SFM). Overall, the astrocytic relative cell proliferation did not change significantly after incubation with the MCM's relative to the control group.

Furthermore, we wanted to analyze if there is a different influence between the melanoma secretome of cutaneous melanoma cells and the one of melanoma brain metastasis cells. Therefore, we compared the relative cell proliferation of astrocytes activated by the cutaneous melanoma cell line CM parental with the ones activated by melanoma brain metastasis cell lines BM-ME5, BM-ME-M2088 and BM-ME-M1731. However, in this case as well, no notable distinction was found between the influence of cutaneous and the brain metastasis melanoma cell lines.

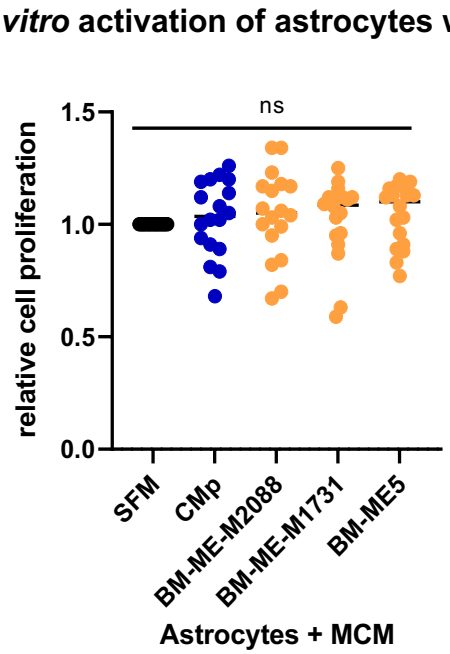


Figure 8: Relative cell proliferation of astrocytes after in vitro activation of astrocytes with MCM. The x-axis reveals the relative cell proliferation of the astrocytes after the incubation with MCM or SFM as a control, while on the y-axis the used MCMs are listed. n = 3.

3.2 Direct contact between astrocytes and melanoma cells results in an increased astrocytic proliferation

To analyze if direct contact between astrocytes and melanoma cells has an impact on the astrocytic proliferation compared to the previously analyzed indirect contact, co-culturing was performed. Every experiment was repeated six times, to validate its reliability.

First, to see if co-culturing of astrocytes and melanoma cells influences astrocytic proliferation, we compared the relative cell proliferation rate of co-cultured astrocytes with the control group. Hereby, co-culturing of astrocytes with CM and BM cell lines showed a notable rise in the astrocytic proliferation compared to the control group ($p < 0{,}0001$).

To further test if a significant difference between cutaneous and brain metastasis melanoma cell lines on the influence of astrocytic proliferation exists, the data of the CM cell line were checked against the BM cell line. Hereby, no significant difference could be observed. Therefore, the increase of astrocytic proliferation was as great with the CM cell line as with the BM cell line.

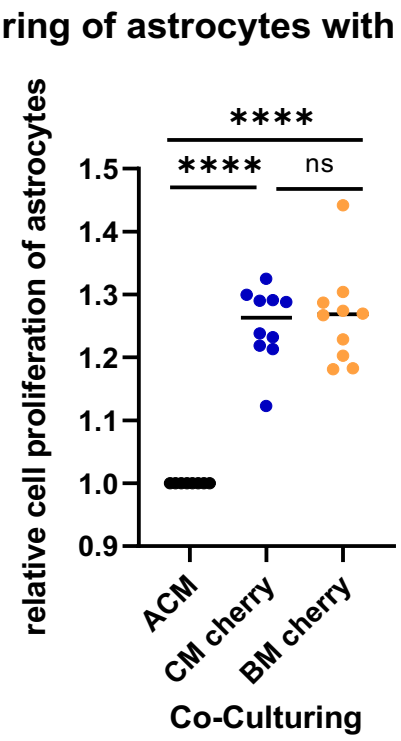


Figure 9: Relative cell proliferation of astrocytes after co-culturing of astrocytes with melanoma cells. The x-axis shows the relative cell proliferation of the astrocytes after co-culturing or only incubation with astrocyte medium as a control, while on the y-axis the co-cultured immunofluorescence-labeled CM and BM cell line and the control with astrocyte medium incubation are listed. n = 6, **** $p < 0{,}0001$.

3.3 Analysis of astrocytic gene expression after *in vitro* activation of astrocytes with MCM

As previously demonstrated the melanoma secretome has no significant influence on astrocytic proliferation and to increase astrocytic proliferation, direct contact between melanoma cells and astrocytes is needed. However, we asked ourselves if the melanoma secretome has an an impact on astrocytic gene expression. Therefore, after *in vitro* activation of astrocytes with melanoma cells conditioned medium, followed by RT-qPCR analysis, we assessed the measurement of mRNA expression fold change, to determine if the melanoma cell secretome has an impact on astrocytic mRNA expression. Each experiment was repeated four times.

As our focus in this thesis was put on the influence of melanoma cells on astrocytic proliferation, the inflammatory tumor microenvironment through the interaction with astrocytes and the chemoattraction of melanoma cells, the focus was put on the following genes: Astrocytic markers such as GFAP, ALDH1 and GLT1, inflammatory factors such as IL-1β, IL-17 and TNF-α, the chemokine CXCL10 and the connexin Cx43.

3.3.1 Melanoma cell secretome influences the astrocytic mRNA expression of the astrocytic marker GLT1

The examination of gene expression in astrocytes indicated that the levels of mRNA expression levels for the astrocytic markers GFAP and ALDH1 remained largely unchanged following the incubation of astrocytes with MCM (Figure 10 A and B). However, the melanoma cell secretome caused a significantly reduced mRNA expression of GLT1 in two out of four melanoma cell lines: the mRNA expression of GLT1 exhibited a notable decrease after the incubation of astrocytes with the BM-ME-M2088 and BM-ME-M1731 conditioned medium ($p < 0,05$) but not after incubation with CM parental or BM-ME5 conditioned medium. However, no significant differences between cutaneous or brain metastasis melanoma cell lines can be observed (Figure 10 C).

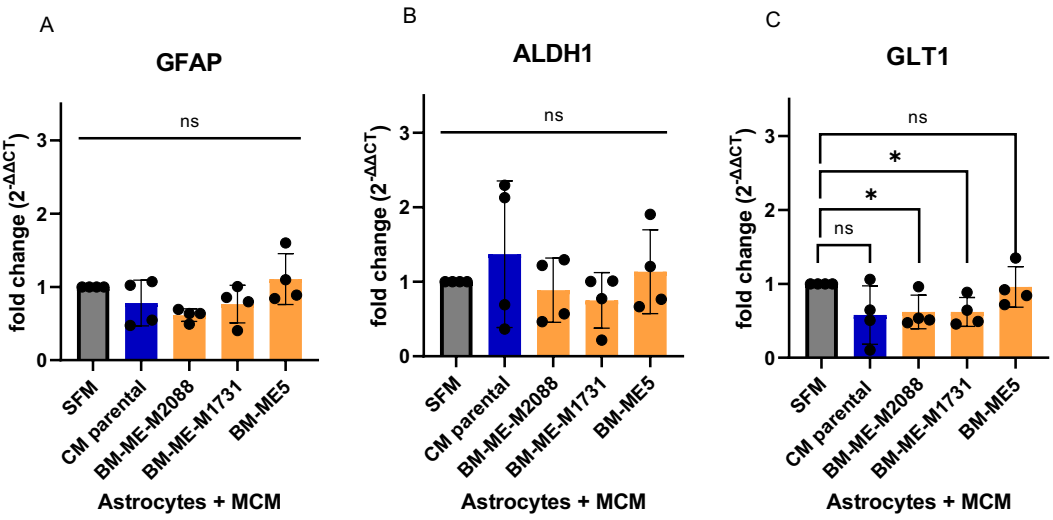


Figure 10: Fold change of astrocytic GFAP (A), ALDH1 (B) and GLT1 (C) mRNA expression after in vitro activation of astrocytes with MCM. The horizontal axis demonstrates the fold change ($2^{-\Delta\Delta CT}$) of the astrocytic mRNA expression after the incubation with the respective MCM or SFM as a control. On the y-axis the used cell lines for the MCMs are listed. n = 4, * $p < 0,05$, ns (non-significant).

3.3.2 Melanoma cells can induce astrocytes to from a tumor-promoting inflammatory microenvironment

To examine if the melanoma cell secretome modulates the mRNA expression of astrocytes regarding inflammation-related factors, the levels of mRNA expression for IL-1β, IL-17 and TNF-α were assessed. The data showed that the incubation of astrocytes with MCM resulted in an upregulation of IL-1β and a downregulation of IL-17 and TNF-α gene expression. IL-1β mRNA expression in astrocytes is markedly upregulated after incubation with CM parental, BM-ME-M2088 and BM-ME5 conditioned medium, but not with BM-ME-M1731 conditioned medium (Figure 11 A). IL-17 mRNA expression is markedly decreased in astrocytes after incubation with all MCM's (Figure 11 B). TNF-α mRNA expression is notably downregulated in astrocytes after incubation with all MCM's (Figure 11 C). Therefore, we could show that the melanoma cell secretome modulates the mRNA expression of inflammatory markers. However, when we compared the data of the cutaneous melanoma cell line with the data of the brain metastasis cell lines, no significant differences could be observed.

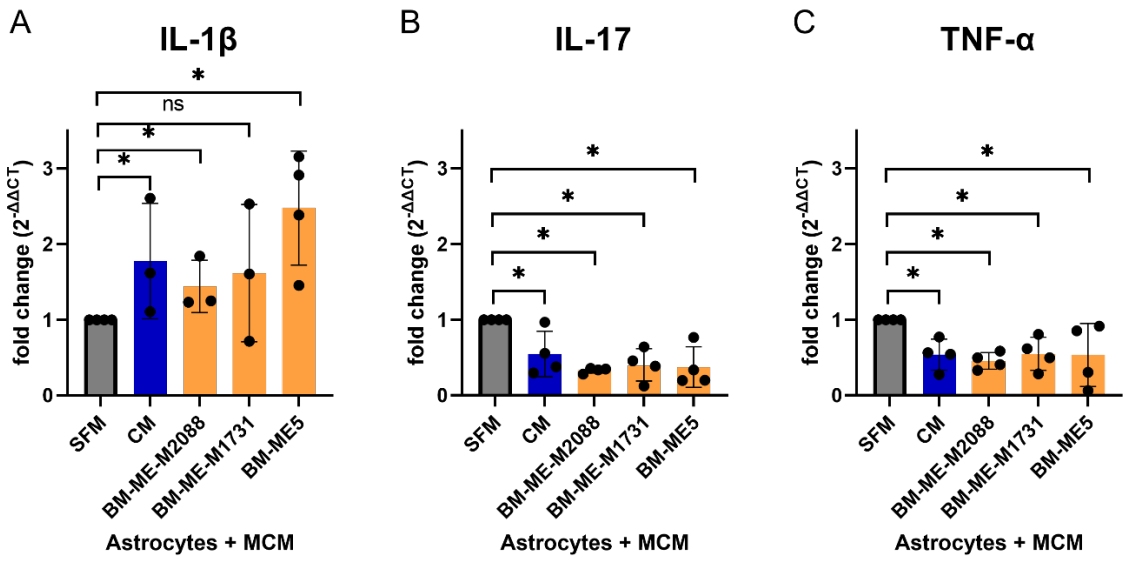


Figure 11: Fold change of astrocytic IL-1β (A), IL-17 (B) and TNF-α (C) mRNA expression after in vitro activation of astrocytes with MCM. The horizontal axis demonstrates the fold change ($2^{-\Delta\Delta CT}$) of the astrocytic mRNA expression after the incubation with the respective MCM or SFM as a control. On the y-axis the used cell lines for the MCMs are listed. n = 4, * p < 0,05.

3.3.3 The melanoma cell secretome does not influence the astrocytic mRNA expression of the chemokine CXCL10

Previous studies demonstrated that CXCL10 that is secreted by astrocytes, attracts melanoma cells towards the brain and therefore supports the formation of brain metastasis. To analyze if its expression by astrocytes is further enhanced by the melanoma cell secretome, the new melanoma cell model was performed as mentioned above.

Our results showed that the incubation of astrocytes with MCM had no impact on the astrocytic gene expression of CXCL10 compared to the control group. These findings indicated that the melanoma cell secretome did not result in the modulation of the astrocytic CXCL10 mRNA expression and that therefore indirect contact between melanoma cells and astrocytes does not have an impact on the astrocytic CXCL10 gene expression (Figure 12).

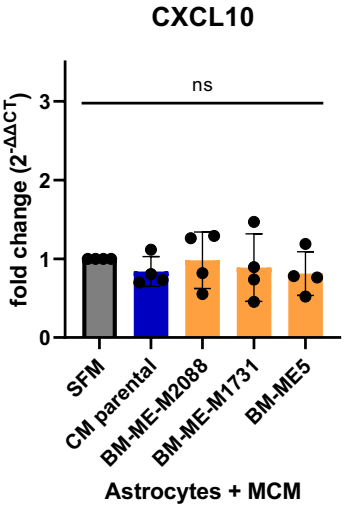


Figure 12: Fold change of astrocytic CXCL10 mRNA expression after in vitro activation of astrocytes with MCM. The horizontal axis demonstrates the fold change ($2^{-\Delta\Delta CT}$) of the astrocytic mRNA expression after the incubation with the respective MCM or SFM as a control. On the y-axis the used cell lines for the MCMs are listed. CXCL10 mRNA expression in astrocytes is not significantly changed after incubation with all MCM's. n = 4

3.3.4 The melanoma cell secretome does not influence the astrocytic mRNA expression of the connexin 43 (Cx43)

Given that Cx43 is the main connexin that allows the communication of melanoma cells with astrocytes in the brain, its mRNA expression was analyzed to deconstruct if it is influenced by the melanoma cell secretome.

However, when comparing the astrocytic gene expression of Cx43 of activated astrocytes with the control group, no significant difference could be observed. Therefore, our data indicates, that there is no significant modulation of the astrocytic Cx43 mRNA expression when astrocytes are exposed to the secretome of melanoma cells (Figure 13).

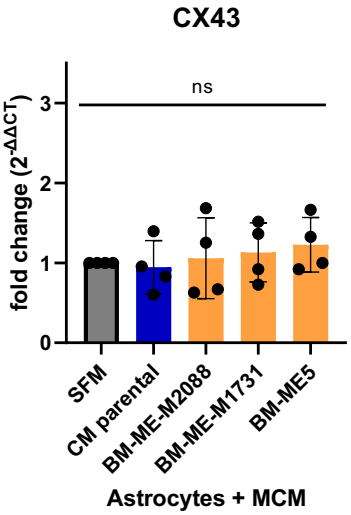


Figure 13: Fold change of astrocytic Cx43 mRNA expression after in vitro activation of astrocytes with MCM. The horizontal axis demonstrates the fold change ($2^{-\Delta\Delta CT}$) of the astrocytic mRNA expression after the incubation with the respective MCM or SFM as a control. On the y-axis the used cell lines for the MCMs are listed. Cx43 mRNA expression in astrocytes is not significantly changed after incubation with all MCM's. n = 4

3.4 Astrocytes induce the migration of melanoma cells

To analyze if astrocytes can stimulate the spread of melanoma cells towards the CNS, *in vitro* migration assays were done. CM parental, BM-ME5 and BM-ME-M1731 cells were primed with ACM to determine an influence of the astrocytic secretome in sight of the migratory potential. Unprimed cells were used as a control. BM-ME-M2088 cells exhibited no migration regardless of the experimental condition. The observations illustrate that the priming of melanoma cells with the astrocytic secretome before the assay had no significant bearing on the migration of all melanoma lines. However, the results indicate that astrocytes significantly enhanced the migratory capacity of all melanoma cells ($p < 0{,}0001$). Migration assays for each melanoma cell line were performed at least twice, to validate its reliability.

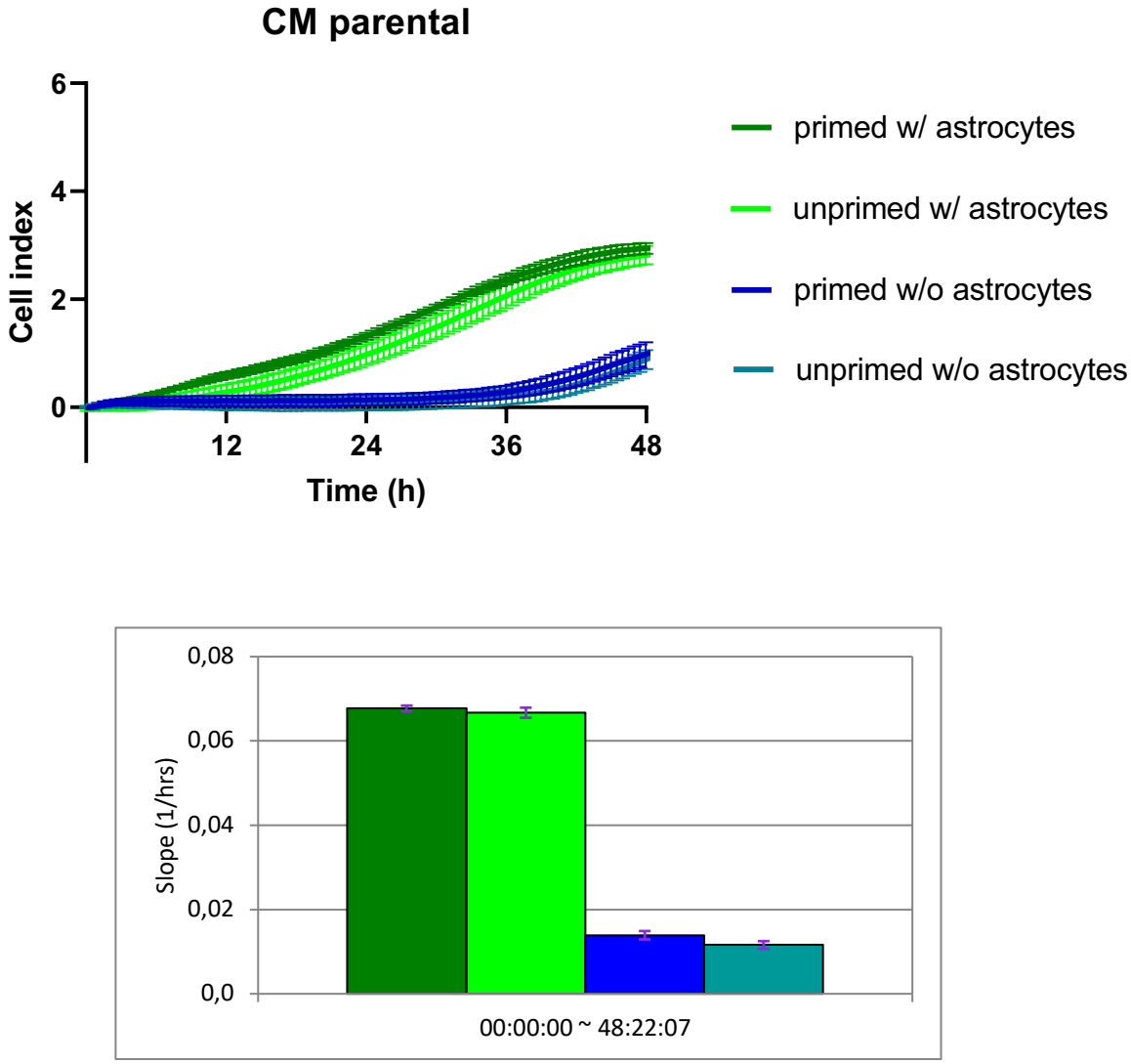


Figure 14: Migration assay of CM parental cells towards astrocytes. A: Graph of CM parental cell migration towards astrocytes (green) and towards DMEM (blue) as a control. The primed CM parental cells (dark green) migrate significantly more towards astrocytes compared to unprimed CM parental cells (* $p < 0,05$). The migration of CM parental cells towards astrocytes is significantly greater compared to the control group with DMEM (**** $p < 0,0001$). B: Slope (1/hrs) of each experiment. n = 2.

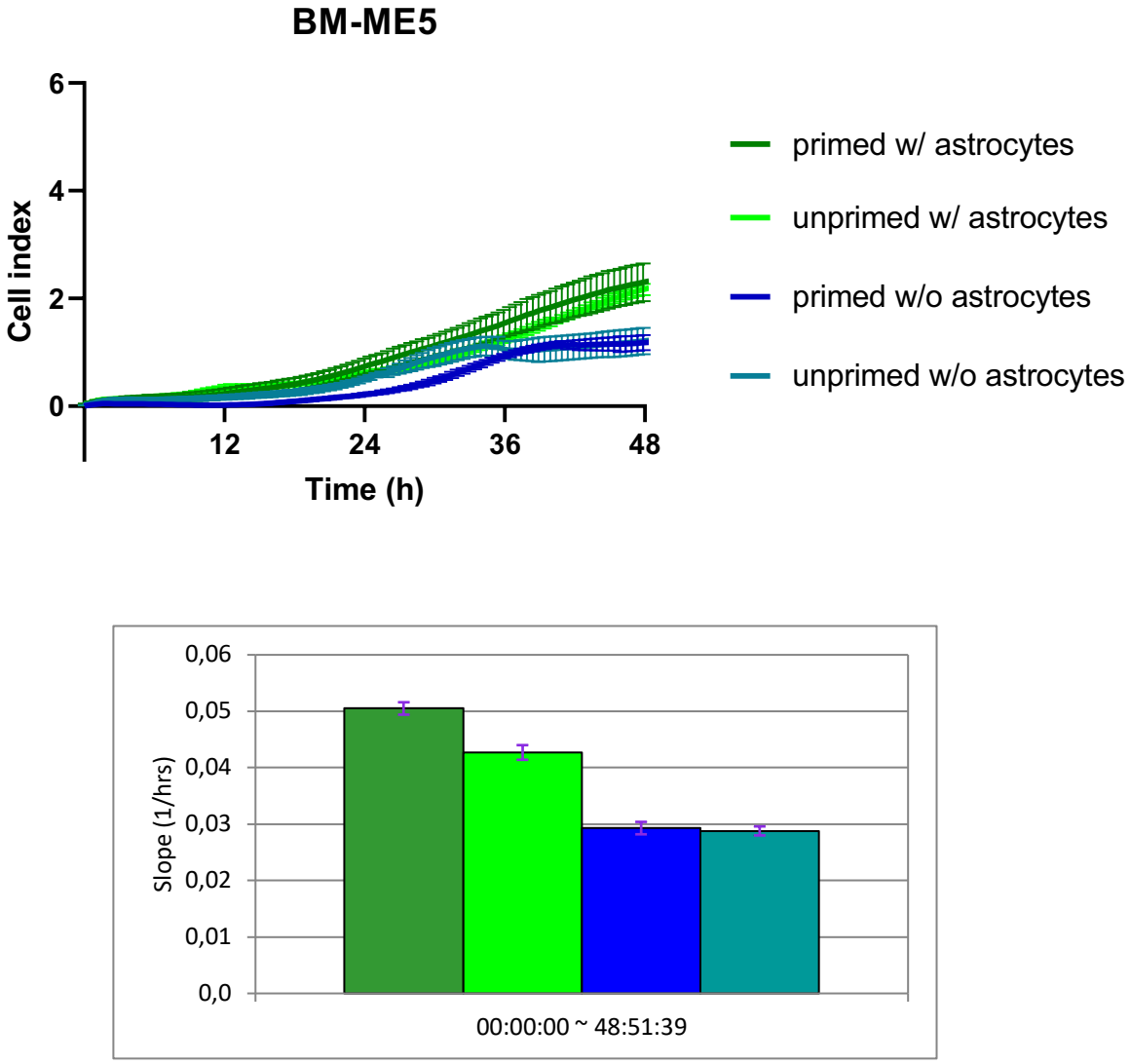


Figure 15: Migration assay of BM-ME5 cells towards astrocytes. A: Graph of BM-ME5 cell migration towards astrocytes (green) and towards DMEM (blue) as a control. The primed BM-ME5 cells (dark green) do not migrate significantly more towards astrocytes compared to unprimed BM-ME5 cells. The migration of primed BM-ME5 cells towards astrocytes is significantly greater in contrast to the primed control group with DMEM (**** $p < 0,0001$). The migration of unprimed BM-ME5 cells towards astrocytes is also markedly elevated when contrasted with the unprimed control group using DMEM, however to a lesser extent (* $p < 0,05$). B: Slope (1/hrs) of each experiment. n = 2.

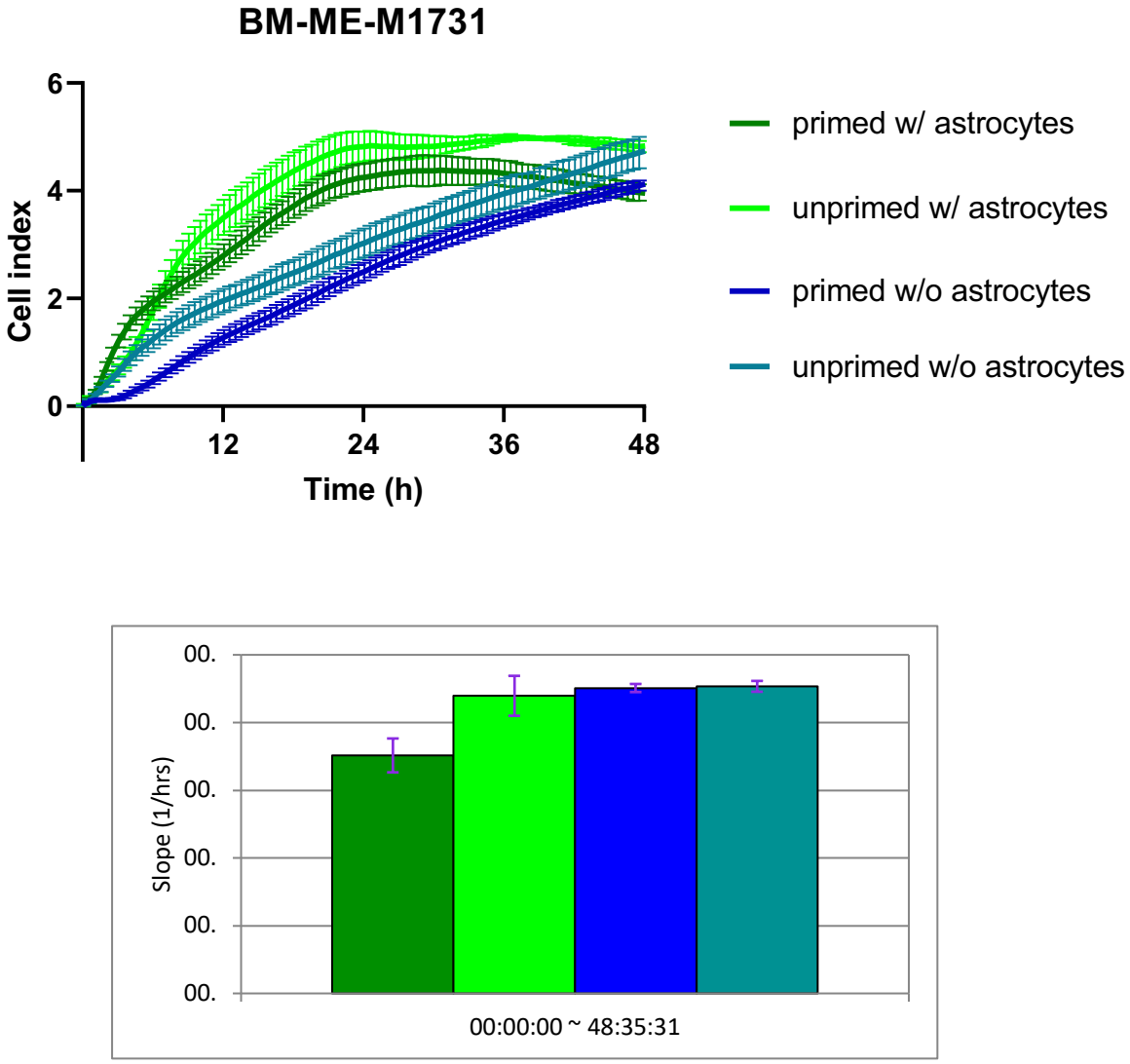
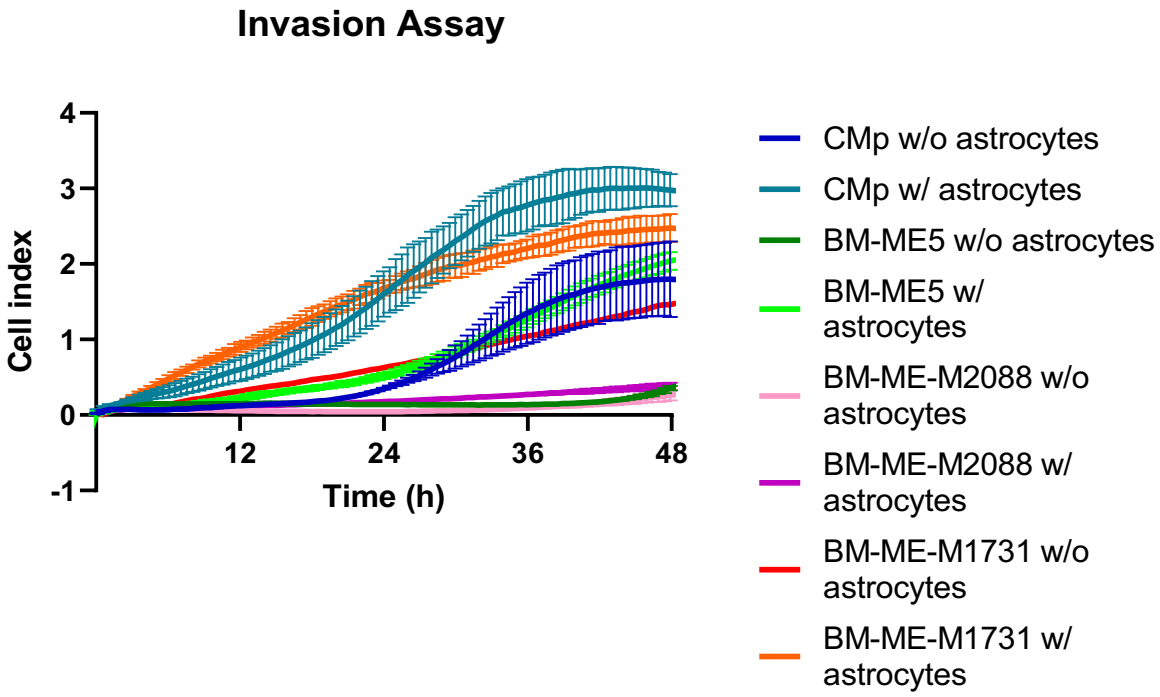


Figure 16: Migration assay of BM-ME-M1731 cells towards astrocytes. A: Graph of BM-ME-M1731 cell migration towards astrocytes (green) and towards DMEM (blue) as a control. The primed BM-ME-M1731 cells (dark green) migrate significantly more towards astrocytes compared to unprimed BM-ME-M1731 cells (**** p < 0,0001). The migration of BM-ME-M1731 cells towards astrocytes is notably elevated in contrast to the control group with DMEM (**** p < 0,0001). B: Slope (1/hrs) of each experiment. n = 2.

3.5 Astrocytes induce the invasive capacity of melanoma cells

To further validate whether astrocytes impact melanoma cell invasion, invasion assays with the melanoma cell lines CM parental, BM-ME5, BM-ME-M1731 and BM-ME-M2088 were performed by using the xCELLigence system. The invasion assays for each melanoma cell line were performed at least twice, to validate its reliability. In concurrence to the migration assay, the cell line BM-ME-M2088 did not show any significant invasion. However, with CM parental, BM-ME5 and

BM-ME-M1731, the presence of astrocytes led to a notably higher invasion of the melanoma cell lines ($p < 0.0001$).



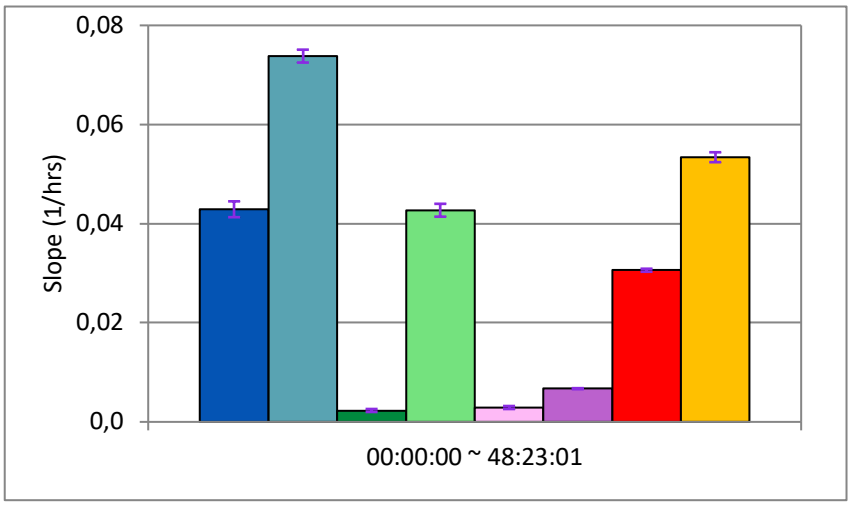


Figure 17: Invasion assay of melanoma cells towards astrocytes. The x-axis demonstrates the cell index of invasion of melanoma cells. The y-axis shows the time in hours. The BM-ME-M2088 does not show any significant invasion, while the melanoma cell lines CM parental, BM-ME5 and BM-ME-M1731 show significantly enhanced migration (**** $p < 0,0001$) when astrocytes were present, in contrast to the control group with DMEM. $n = 2$, **** $p < 0,0001$.

4. Discussion

The advancement of immune checkpoint inhibitors (ICI) has improved the medical care of melanoma patients. However, melanoma patients with a diagnosis for melanoma brain metastasis still show a median survival rate of only 1-2 years (37). Therefore, it is essential to identify new biological methods involved in brain metastasis formation to overcome these challenges and to be able to further develop new therapeutical strategies. Previous studies have already described that astrocytes contribute to the establishment of melanoma brain metastasis (1,81,83,84). Nonetheless, the precise bidirectional relationship between melanoma cells and astrocytes still needs to be further addressed. The objective of this thesis is to further analyze the reciprocal interaction between melanoma cells and astrocytes. For this purpose, we looked at the impact of melanoma cells via direct and indirect contact on astrocytes and how this modulates the astrocytic cellular functions. Furthermore, we also had a look if astrocytes have an impact on the migration and the infiltration of melanoma cells into the brain.

Izraely et al. could show that melanoma cells have significant effects on microglia cells, including morphological changes and enhanced cell proliferation and migration (157). Therefore, we conjectured that melanoma cells might have a similar effect on astrocytes. Our data indicates that *in vitro* activation of astrocytes with MCM does not increase astrocytic proliferation. However, we know that through gap junctions, astrocytes can also be in direct contact with melanoma cells (83). Therefore, we did co-culturing of astrocytes with melanoma cells to analyze the influence on astrocyte proliferation with direct contact. The co-culturing of astrocytes with melanoma cells showed a significant enhanced proliferation of astrocytes. Therefore, we could show that direct contact between astrocytes and melanoma cells is needed, for melanoma cells to be able to modulate the cellular function regarding proliferation of astrocytes.

In conclusion, we can say that with direct contact, melanoma cells have an even stronger effect on reprogramming astrocytes and therefore inducing tumor-supporting changes in the TME, than with only indirect contact with astrocytes. However, our RT-qPCR results also demonstrate that direct contact is not necessarily needed for melanoma cells to modify the gene expression of astrocytes, in contrast to the modulation of astrocytic proliferation. The melanoma cell secretome already results in the modulation of astrocytic mRNA expression, which could benefit tumor progression. Certainly, in agreement to our results of the Ki-67 immunofluorescent staining of astrocytes, direct contact could result in an even enhanced modulation.

Alterations in the TME form a welcoming environment for metastasis by the modification of the expression of tumor favorable proteins (5). Klein et al. demonstrated that human brain-metastasizing melanoma cells can induce a transformation in astrocytes, leading to the generation of inflammatory promoting factors, including IL-23, and that the interactions via paracrine signaling amidst astrocytes and melanoma cells are mutually influential (81). Therefore, with RT-qPCRs we wanted to identify further effects of the melanoma cell secretome on alterations of the astrocytic gene expression. With the results of astrocytic mRNA expression levels after in vitro activation of astrocytes, we could show that the melanoma cell secretome has no influence on the

astrocytic gene expression of the chemokine CXCL10 or the connexin Cx43, a small impact on the astrocytic marker GLT1 and a great impact on inflammatory cytokines.

The melanoma cell secretome did not momentously influence the astrocytic gene expression of GFAP and ALDH1, but of GLT1. As already mentioned, upon the formation of brain metastasis, astrocytes become active. Hereby, in contrast to GFAP and ALDH1, GLT1 is downregulated during reactive astrogliosis (105). This stands in agreement to our results, that the GLT1 expression is reduced in astrocytes when they get in contact with the melanoma cell secretome. As glutamate is a major bioenergy substrate for cancer cell growth, decreased GLT1 expression would result in an increased glutamate concentration in the extracellular space, which could then be used by the tumor as a bioenergy substrate (122). Therefore, decreased GLT1 expression could result in an increase of tumor growth. Additionally, it has been previously stated by Haowei Yi *et al.* that excessive extracellular glutamate can activate glutamate receptors on cancer cells resulting in malignant growth (122). However, the GLT1 expression in our experiments was only reduced in two out of four melanoma cell lines. However, as mentioned above, a direct contact between melanoma cells and astrocytes may probably show an even more significant downregulation of GLT1 expression in astrocytes and may even show an upregulation of GFAP and ALDH1 which we would expect as both are upregulated in reactive gliosis (94,105).

Furthermore, our results indicated that the melanoma cell secretome particularly influences the gene expression of inflammatory markers. Reactive astrocytes secrete various cytokines that contribute to the innate immune response and promotes tumor growth and invasion (123,124). Our results exhibit an upregulated astrocytic mRNA expression of IL-1β and a downregulated astrocytic mRNA level of IL-17 and TNF-α.

An upregulation of astrocytic IL-1β secretion could result in an increased activation of its main signaling pathways - the NF-kB and MAP kinase signalling cascade - through which IL-1β mainly triggers signaling pathways that promote cell survival and proliferation (143). This would therefore generally promote cancer progression. Even though IL-1β has been described to be required for melanoma metastasis, invasiveness, and angiogenesis, it also has been documented to exhibit certain anti-tumorigenic effects (133,143). As IL-1β is believed to be mainly secreted by tumor-associated macrophages, it is not known how big the impact of astrocytic IL-1β secretion on the TME really is. However, we could show that the IL-1β in the TME is partly secreted by astrocytes. As in a murine model, treatment of the mice with an IL-1β antagonist resulted in reduced tumor growth and lung metastasis. Therefore, IL-1β antagonism might probably be a therapeutic option for handling metastatic tumors in the future (134).

In addition, IL-17 is recognized for its involvement in pro-tumorigenic and anti-tumorigenic effects (140). For example, a downregulation of IL-17 could benefit the tumor by resulting in a decrease of $CD4^+$ and $CD8^+$ T cell and dendritic cell recruitment, decrease in NK cell activity, decrease of the generation and activation of cytotoxic T lymphocytes, and decrease in neutrophil infiltration (140). However, because this would also lead to decreased proliferation, increased apoptosis, increased susceptibility to immune response through decreased recruitment of MDSCs and reg-

ulatory T cells, decreased angiogenesis and metastasis, there would also be some pro-tumorigenic effects of IL-17 (140). Consequently, it is important to further survey the functions of IL-17 in the TME to develop new potential therapeutic strategies that could address the IL-17 signalling and inhibit tumor growth and progression.

As TNF-α has a crucial role in apoptosis and necrosis which can prevent cancer. A downregulation of TNF-α could mean that there is less apoptosis and therefore enhanced tumor proliferation (158). As mentioned in the introduction, unlike its tumor-inhibiting properties, it is also associated with a range of tumor-promoting processes, for example in the initiation of MAPK and NF-kB signalling cascades (158). In agreement to our hypothesis and results, a study performed *in vivo* using BL6 mice has demonstrated that the most malignant cancers express low levels of TNF-α (159). Interestingly, immunotherapy increases TNF-α levels, which correlates with significant enhancement of overall survival (160). Overall, TNF-α most likely has an anti-tumorigenic effect, and it therefore would favor the tumor, when astrocytic TNF-α would be reduced.

We can therefore conclude that melanoma cells can induce astrocytes to establish a tumor-promoting inflammatory microenvironment. However, the mentioned inflammatory cytokines have multiple pathways and effects on the TME and only few are known until now. Therefore, we can only speculate how they could promote the inflammatory TME. Furthermore, it is important to consider that they all also exhibit some tumor-inhibiting effects. Certainly, the changes in the astrocytic expression of inflammatory cytokines through the melanoma cell secretome, might serve as a significant factor participating in the emergence of treatment resistance against immunotherapy in individuals with melanoma brain metastasis. Therefore, addressing this astrocytic response to melanoma cells is important to find new potential therapeutic targets, to enhance therapy response.

Concordantly, we observed no substantial alteration in the astrocytic mRNA expression of the chemokine CXCL10 or the connexin Cx43 after indirect contact. However, this might be different with direct contact of melanoma cells with astrocytes. As astrocytic CXCL10 secretion is increased upon reactive astrogliosis and has been shown to promote the chemoattraction of melanoma cells towards the brain, a melanoma cell-induced increase in CXCL10 expression could further support brain metastasis formation (1). Moreover, as Cx43 enables melanoma cells to directly communicate with astrocytes, an increased astrocytic Cx43 gene expression might result in increased contact and communication between melanoma cells and astrocytes (83). This could then result in an enhancement of the tumor-promoting reprogramming effect of melanoma cells on astrocytes.

As we only looked at the astrocytic mRNA expression, the next step would be to analyze the cytokine levels on a post-translation level by analyzing the protein levels of the cytokines and afterwards, to assess the actual cytokine secretion. Summing up, we can say that the melanoma cell secretome has an impact on astrocytic gene expression, particularly on its inflammatory markers. However, it would be intriguing to evaluate astrocytic mRNA expression after co-culturing astrocytes with melanoma cells, to see if this results in an increased modulation, including the

expression of astrocytic markers, the chemokine CXCL10 and the connexin Cx43 and afterwards, to assess the cytokines protein levels and secretions.

Despite the fact that we did not find any differences regarding astrocytic chemokine or connexin gene expression, we performed an *in vitro* study to ascertain whether cytokines do have an impact on melanoma cell migration and invasion and if this could provide a future prospective for the study of other molecules. With migration and invasion assays, we analyzed if astrocytes have an impact on the movement and penetration of melanoma cell into the brain tissue. Initially we hypothesized that priming of melanoma cells using astrocyte conditioned medium might further enhance migration. However, our experiments showed that the priming of melanoma cells using astrocyte conditioned medium did not result in an enhanced movement of melanoma cells towards the brain. As melanoma cells that metastasize are not directly exposed to the astrocyte secretome before entering the brain parenchyma, this stands in agreement with our results. All melanoma cell lines but the BM-ME-M2088 cell line showed migration towards astrocytes. The BM-ME-M2088 cell line behaves differently in the cell culture as it also grows in the supernatant and in most cases shows the least impact in the astrocytic mRNA expression when used for the MCM, we concluded that these characteristic differences fit to the results of the migration and invasion assays. The data suggests that astrocytes significantly enhance melanoma cell migration to the brain and therefore probably has an impact on the directed migration of melanoma cells towards the brain as it has been illustrated with the chemokine receptor CCR7 (63). We concluded that the initial exposure of melanoma cells to the astrocyte secretome, directly results in enhanced migration towards the brain and that priming is not necessary. Furthermore, our data is supported by the fact that astrocyte-secreted CXCL10 results in an astrocyte-induced chemoattraction of melanoma cells towards the brain, demonstrated by Doron et al. (1). However, several other signaling pathways in reciprocal interaction are most likely involved and further investigation is needed to detect other possible ways, how astrocytes can chemoattract melanoma cells towards the brain.

To further address if astrocytes also have an impact on melanoma cell invasion, invasion assays were performed. Concordantly to the migration assay, we observed that astrocytes enhanced melanoma cell invasion. In agreement to the migration assays, the BM-ME-M2088 cell line also exhibited no invasion towards the astrocytes. However, all other melanoma cell lines did, therefore we can say that astrocytes do have an impact on the invasion of melanoma cells into the brain tissue. Validation of clinical translation of the findings to investigate if astrocytes also simplified the invasion of melanoma cells inside the brain tissue *in vivo*, which would therefore facilitate melanoma brain metastasis formation, would be very interesting. As we now know that astrocytes enhance melanoma cell migration and invasion, the focus can now be put on further possible cellular mechanisms that are responsible for this enhancement.

As previously mentioned, it is already known that metastatic melanoma cells exhibit a specific organ tropism which is for instance marked by the expression of specific surface receptors, as the chemokine receptor CCR7 on melanoma cells supports lymph node and brain metastasis, the

receptor CXCR4 promotes lung metastasis and the receptor CXCR3 again supports brain metastasis (1,62,63). Due to the development of those characteristics of melanoma cells, which depend on the organ they metastasize to, we initially hypothesized that melanoma brain metastasis cells may exhibit a stronger effect on astrocytic alteration compared to the cutaneous melanoma cell line. However, if we compare the data of melanoma brain metastasis cells with the cutaneous melanoma cell line of each experiment, we cannot see a significant difference between them. From previous experiments, we know that our used CM parental cell line is a quite aggressive one, therefore it would be interesting if other cutaneous melanoma cell lines would exhibit a difference to melanoma brain metastasis cells and may for example have less impact on the astrocytic mRNA expression. To further address this hypothesis, it would be revealing to include more cutaneous melanoma cell lines.

Finally, to determine if the results of our murine model are translatable to humans, validation of clinical translation of the findings by analyzing human brain metastasis would be very intriguing. Furthermore, it would be important to analyze the consequence that reactive astrocytes have on metastatic melanoma cell lines in the brain. Hence, it would also be very interesting to do RT-qPCRs of melanoma cells after in vitro activation with astrocyte conditioned medium and after co-culturing with astrocytes.

To sum up, we can conclude that a lot about the reciprocal interaction between melanoma cells and astrocytes remains unknown and further research is needed to find potential targets for possible therapeutic interventions. Inhibiting the interaction between melanoma cells and astrocytes might prevent those tumor-promoting properties of astrocytes and finding potential therapeutic targets could possibly decrease the amount of melanoma brain metastasis formation and on the other hand enhance therapy response to immunotherapy. Until now, there are few effective treatment options for melanoma brain metastasis. Also, only a few astrocyte-based therapies treating brain metastasis such as Macitentan and Gap junction inhibitors exist that are able to increase cancer cell chemosensitivity and to significantly decrease lung and breast cancer brain metastatic tumor burden (83,85). Therefore, it is essential to do further research about melanoma brain metastasis to be able to discover new targeted therapy options to improve its prognosis and overall survival.

5. Conclusion

Melanoma brain metastasis develops in approximately half of advanced-stage melanoma patients and remains a diagnosis with a poor prognosis. In the TME, melanoma cells are in complex interactions with astrocytes. When astrocytes come in contact with melanoma cells, they can have pro-metastatic effects by releasing certain molecules that affect activity and gene expression of surrounding cells including the immunological microenvironment. The intent of this dissertation thesis was to figure out the mutual interaction between melanoma cells and astrocytes by analyzing its influence on the cellular function of astrocytes, including proliferation, migration, invasion, and further its gene expression. We hypothesized that melanoma cells increase astrocytic proliferation after indirect or direct contact and that they might influence astrocytic gene expression. Furthermore, we hypothesized that, vice versa, astrocytes have the potential to impact melanoma cell migration and invasion. For this analysis, melanoma cell lines obtained from different metastatic sites of the MT/*ret* animal model were used and immunofluorescent staining using Ki-67, RT-qPCRs, migration, and invasion assays were performed.

We could show that a direct contact between astrocytes and melanoma cells is needed for a melanoma cell-induced increase of astrocyte proliferation. However, indirect contact through the melanoma cell secretome results in a modulation of astrocytic gene expression particularly of inflammatory cytokines. In addition, we could show that astrocytes increase melanoma cell migration and invasion and therefore significantly facilitate the emergence of melanoma brain metastases. In the TME, melanoma cells can induce tumor-promoting properties in astrocytes, such as the formation of a tumor-promoting inflammatory microenvironment. Through this modulation, melanoma cells can induce astrocytes to establish a tumor-promoting inflammatory microenvironment. Inhibiting the interaction between melanoma cells and astrocytes could prevent these tumor-promoting properties of astrocytes. Finding potential therapeutic targets in this connection could potentially reduce the emergence of melanoma brain metastases and improve the response to immunotherapy.

Literature Review

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Supplement

Supplement 1: Data of migration assay of the CM parental cell line. The table shows the corresponding slope, standard deviation (SD), amount of seeded CM parental cells and if the migration was done with astrocytes or DMEM (as a control).

Tumor cell	Slope (1/hrs)	SD	Cell number	Treatment
Primed CM parental	0,0677	0,0007	60000	Astrocytes
Unprimed CM parental	0,0667	0,0012	60000	Astrocytes
Primed CM parental	0,0139	0,001	60000	DMEM
Unprimed CM parental	0,0116	0,0009	60000	DMEM

Supplement 2: Data of migration assay of the BM-ME5 cell line. The table shows the corresponding slope, standard deviation (SD), amount of seeded BM-ME5 cells and if the migration was done with astrocytes or DMEM (as a control).

Tumor cell	Slope (1/hrs)	SD	Cell number	Treatment
Primed BM-ME5	0,0505	0,0011	60000	Astrocytes
Unprimed BM-ME5	0,0427	0,0013	60000	Astrocytes
Primed BM-ME5	0,0293	0,0011	60000	DMEM
Unprimed BM-ME5	0,0288	0,0008	60000	DMEM

Supplement 3: Data of migration assay of the BM-ME-M1731 cell line. The table shows the corresponding slope, standard deviation (SD), amount of seeded BM-ME-M1731 cells and if the migration was done with astrocytes or DMEM (as a control).

Tumor cell	**Slope (1/hrs)**	**SD**	**Cell number**	**Treatment**
Primed BM-ME-M1731	0,0703	0,005	60000	Astrocytes
Unprimed BM-ME-M1731	0,0879	0,0059	60000	Astrocytes
Primed BM-ME-M1731	0,0902	0,0012	60000	DMEM
Unprimed BM-ME-M1731	0,0907	0,0016	60000	DMEM

Supplement 4: Data of invasion assay of the melanoma cell lines. The table shows the corresponding slope, standard deviation (SD), amount of seeded melanoma cells and if the migration was done with astrocytes or DMEM (as a control).

Tumor cell	**Slope (1/hrs)**	**SD**	**Cell number**	**Treatment**
CM parental	0,0429	0,0016	60000	DMEM
CM parental	0,0738	0,0013	60000	Astrocytes
BM-ME5	0,0023	0,0003	60000	DMEM
BM-ME5	0,0427	0,0013	60000	Astrocytes
BM-ME-M1731	0,0029	0,0003	60000	DMEM
BM-ME-M1731	0,0067	0,0001	60000	Astrocytes
BM-ME-M2088	0,0306	0,0003	60000	DMEM
BM-ME-M2088	0,0534	0,001	60000	Astrocytes

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Affidavit

Eidesstattliche Versicherung

Schönherr, Rebecca

Name, Vorname

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel:

Reciprocal interaction of malignant melanoma brain metastasis with cells of the tumor microenvironment.

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, 08.05.2023 — Rebecca Schönherr

Ort, Datum — Unterschrift Doktorandin bzw. Doktorand

List of publications

1. Schrecker C, Behrens S, Schönherr R, Ackermann A, Pauli D, Plotz G, Zeuzem S, Brieger A, SPTAN1 expression predicts treatment and survival outcome in colorectal cancer. Cancers. 2021 Jul 20;13(14):3638.