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Glioblastoma cell-derived apelin has differential effects on myeloid cell migration

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Content

Zusammenfassung

Das Glioblastom (GBM) stellt den häufigsten malignen Hirntumor dar und betrifft pro Jahr in Europa etwa sechs von 100.000 Erwachsenen. Aggressive Interventionen zur Behandlung des Tumors umfassen die Chirurgie, Chemo- und Radiotherapie. Dennoch ist die Ein-Jahres-Überlebensrate mit 20% gering. GBMs charakterisieren sich durch sehr invasives Wachstum mit ausgeprägter Vaskularisation. Die Tumormasse besteht neben Tumorzellen auch aus Immunzellen. Diese tragen zur Bildung eines lokalen Milieus bei, das von den Tumorzellen für deren Wachstum und zur Infiltration anderer Hirnregionen genutzt wird. Tumorassoziierte Mikroglia und Makrophagen (TAM) repräsentieren bis zu 50% dieser Nicht-Tumorzellen in der Mikroumgebung von GBM, weshalb sie ein mögliches Ziel für zukünftige Therapien darstellen. Im GBM Mausmodell fand unsere Arbeitsgruppe unter der Leitung von *Dr. Roland Kälin* Hinweise auf eine signifikant erniedrigte TAM-Dichte im Umfeld von Gliomen deren APLN-Expression ausgeschaltet worden war. Hieraus resultierte die Hypothese, der Apelin-Apelinrezeptor (APLN/APLNR)-Signalweg könnte für die Rekrutierung myeloider Zellen durch GBM von Bedeutung sein.

In dieser Doktorarbeit wird der APLN/APLNR-Signalweg im Kontext der Interaktion von GBM und TAM untersucht. Ich zeige eine direkte Korrelation zwischen starker Expression von APLN in GBM und einer entsprechend erhöhten Dichte von TAM in der Mikroumgebung des Tumors auf. Zudem demonstriere ich, dass APLNR sowohl von BV2 Mikroglia als auch von J774 Makrophagen exprimiert wird. Eine immunologische Stimulation dieser Zellen mit Lipopolysaccharid (LPS) – zur Simulation einer Entzündungsantwort – resultiert in einer niedrigeren Expression des Rezeptorgens. Der APLN/APLNR-Signalweg scheint im Rahmen dieser Entzündungsantwort daher keine besondere Bedeutung zu haben.

Ich zeige auch, dass isoliertes Apelin-13 Peptid zwar die Migration von Mikroglia signifikant erhöht, jedoch auf die Mobilisation von Makrophagen keine größere

Auswirkung hat. Für eine bessere Simulation der *in vivo* Situation verwende ich statt isoliertem Apelin-13 den Zellüberstand von kultivierten Gliomzellen. Ich vergleiche Medium von APLN-Wildtyp-Gliomzellen (WT) mit Medium von APLN-knockdown-Zellen (KD). Hierbei zeigen Mikroglia im Gegensatz zu Makrophagen ein überwiegend verändertes Wanderungsverhalten. Hohe APLN-Expression des Tumors führt zu einer chemo-attraktiven Wirkung auf BV2 Zellen. Daher vermute ich, dass verschiedene GBM Zellen unterschiedliche Signalwege zur Rekrutierung von Mikroglia nutzen – der APLN/APLNR-Signalweg wäre insofern vor allem für Tumore mit hoher APLN-Expression von Bedeutung. Für Makrophagen konnte ich keinen direkten Einfluss von Apelin auf deren Wanderungsverhalten zeigen.

Im Kontext von GBM wirkt APLN unterschiedlich auf Mikroglia und Makrophagen. Verschiedene GBM Zellen und deren APLN Expression scheinen das Verhalten von TAM auf unterschiedliche Weise zu beeinflussen. Es werden weitere Studien nötig sein, um die genaue Bedeutung des APLN/APLNR-Signalweges für die Rekrutierung vom TAM durch GBM offenzulegen. Für einige GBM Zellen scheint er jedoch von entscheidender Relevanz zu sein, womit er auch ein potenzielles Ziel zukünftiger Therapieansätze darstellt.

Werden Mikroglia und Makrophagen dem Kulturmedium verschiedener GBM Zellen ausgesetzt, so zeigt Mikroglia eine geringere Mobilität und Makrophagen eine gesteigerte. Immunhistologisch kann außerdem eine direkte Korrelation von APLN-Expression der GBM-Zellen und der Dichte an TAM im Gehirn gezeigt werden. Gemeinsam führen die Beobachtungen zu der Vermutung, dass Makrophagen im unmittelbaren Umfeld der Gliome die vorherrschenden myeloiden Zellen sind. Um dies jedoch ganz schlüssig zu beweisen, sind weitere Experimente mit einer immunhistologischen Differenzierung zwischen Mikroglia und Makrophagen notwendig.

Abstract

Glioblastoma (GBM) is the most common malignant brain tumor affecting approximately six people every 100.000 adults in Europe every year. Aggressive interventions like surgery, chemo- and radiotherapy have been established to treat the tumor, nevertheless the one-year survival rate is only 20%. GBMs are characterized as very invasive tumors with abundant vascularization. Their microenvironment was shown to include immune cells. These cells create a local milieu, which is used by neoplastic cells to grow and infiltrate other brain regions. Tumor-associated microglia and macrophages (TAM) represent up to 50% of these non-tumor cells in the microenvironment of GBM and therefore represent an interesting target for future therapies. In GBM mouse models our group – led by *Dr. Roland Kälin* – found a significant reduction of TAM density when APLN expression was knocked out in GBM cells. We considered apelin-apelin receptor (APLN/APLNR)-signaling might be part of GBM myeloid cell recruitment.

In this study I investigated the role of APLN/APLNR-signaling in the context of GBM cell interaction with TAM. I found a direct correlation of APLN expression levels and the density of TAM in the microenvironment of GBM. Furthermore, I showed APLNR to be expressed in BV2 microglia and J774 macrophages. Immunological stimulation of these cells with lipopolysaccharide (LPS) to simulate an inflammatory reaction resulted in a lower expression of the receptor gene. I considered APLN/APLNR-signaling to have a low relevance in the context of a typical inflammatory response.

I demonstrated that the apelin-13 peptide is chemoattractive for microglia but not for macrophages. To improve the simulation of the *in vivo* situation, I used glioma supernatant instead of recombinant apelin-13. Under these conditions I compared APLN wildtype (WT) and knockdown (KD) GBM cells. GBM-derived apelin affected migration of microglia stronger than migration of macrophages. I considered that GBM use various pathways – amongst them APLN/APLNR-signaling – to attract microglia towards the tumor. Microglia is attracted especially by strong APLN

expressing GBM. For macrophages I could not show a significant influence of apelin on cell migration.

In the context of GBM, APLN acts in different ways on microglia and macrophages. Different GBM subtypes and their individual APLN expression levels have distinct effects on TAM. Further studies are required to fully disclose the role of APLN/APLNR-signaling in TAM recruitment of GBM. Nevertheless, APLN/APLNR-signaling seems to be a relevant pathway in this process for some glioma cells and therefore represents a potential therapeutic target in the future.

When microglia and macrophages are exposed to different GBM-conditioned medium, microglia show reduced mobility and macrophages increased mobility. In addition, a direct correlation between APLN expression of GBM cells and the density of TAM in the brain can be shown immunohistologically. Together, the observations suggest that macrophages are the predominant myeloid cells in the immediate vicinity of gliomas. However, to prove this, further experiments with an immunohistological differentiation between microglia and macrophages are necessary.

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List of abbreviations

APLN	Apelin
APLNR	Apelin receptor
CNS	Central nervous system
CSF	Colony stimulating factor
GBM	Glioblastoma multiforme
GFP	Green fluorescent protein
IDH	Isocitrate dehydrogenase
IBA1	Ionized calcium-binding adapter molecule 1
IFNγ	Interferon γ
IL	Interleukin
KD	Knock down
KO	Knock out
PDGF	Platelet-derived growth factor
(pyr^{1})apelin-13	Pyroglutamylated apelin-13
qPCR	Quantitative polymerase chain reaction
TAMs	Tumor-associate myeloid cells
TMZ	Temzolomide
VEGF	Vascular endothelial growth factor
WHO	World health organization
WT	Wild type

1. Introduction

1.1 Glioblastoma multiforme

1.1.1 Classification and epidemiology of glioblastoma

Glioblastoma (GBM) is a malignant glioma with an incidence rate of 6/100.000 in Europe [1]. It was responsible for 14,9% of all brain and central nervous tumors diagnosed in the United States from 2009 to 2013 representing the main subtype of malignant brain tumors [2]. It can be found more frequently in adults than children showing the highest incidence rate in patients between 75 and 84 years [2]. The median age of diagnosis for GBM is 64 years and it is more common in males than in females [2]. Patients with a diagnosed glioblastoma show a 1-year survival rate of 20% [3]. After five years only 4,7% are still alive [2]. In 2021, a new WHO classification for tumors of the central nervous system (CNS) was established. It defines four general groups of diffuse gliomas: adult-type diffuse gliomas, pediatric-type diffuse low-grade gliomas, pediatric type diffuse high-grade gliomas and circumscribed astrocytic gliomas. Beside the histological classification, there is another classification based on the malignancy of gliomas. This system differentiates between glioma grade 1 – which are considered to be benign – grade 2 and 3-gliomas and grade 4. GBMs belong to the last group [4]. With the WHO classification of 2016, molecular parameters like the isocitrate dehydrogenase (IDH) mutation status were added to the histology of glioma in order to improve diagnostic and prognostic accuracy as well as the treatment of the patients. The WHO classification of 2021 adds new molecular patterns. Adult-type diffuse gliomas are grouped depending on the IDH mutation status. IDH-wildtype glioblastoma (WHO Grade 4) are identified either by histological parameters like the presence of microvascular proliferation or necrosis or by molecular features such as telomerase reverse transcriptase (TERT)-promoter mutation, epidermal growth factor receptor (EGFR) amplification and combined chromosome 7 gain/chromosome 10 loss (+7/-10) [5] [6]. IDH-mutant astrocytomas are graded WHO 4 when homozygous deletion of cdkn2a/b status can be detected [6]. IDH-wildtype GBMs occur frequently with patients over 55 years of age, whereas IDH-mutation is common in younger patients with GBMs that develop out of lower graded gliomas [7]. *SongTao et al.* showed that IDH-mutation plays an important role

in predicting the benefit of temozolomide treatment, as IDH-mutated glioma respond much better than IDH-wildtype glioma [8].

1.1.2 Etiology of glioblastoma

The etiology of GBMs remains unclear. Confirmed risk factors are ionizing radiation, hereditary syndromes and genetics, ethnicity as well as male sex. However, no effect of mobile phone use, smoking or alcohol consumption on glioma development could be found so far [9] [10].

1.1.3 Histopathology of the tumor

Glioblastoma are mostly located in the cerebral hemispheres and can grow very invasively. The tumor grows heterogeneously with cystic and solid areas on the one and hemorrhage as well as necrosis on the other hand [11]. Under the microscope an extremely pleomorphic cell population with small and poorly differentiated tumor cells as well as large multinucleate cells can be observed. Many of them show a strong mitotic activity. One of the typical characteristics of GBMs is the necrotic area [12]. The extend of this area was shown to be associated with a poor clinical outcome. Moreover, the histological examination shows strong vascular endothelial proliferation in many patients [11].

1.1.4 Pathogenesis of glioblastoma

In the last years, improvements in molecular diagnostics led to more detailed knowledge of GBM pathogenesis [13]. Former studies showed that loss of heterozygosity of chromosome 10q can be detected in 70% of GBMs [14]. Analysis of the underlying genetic lesions showed three different pathways to be most relevant in GBM pathogenesis. Abnormalities in signaling of the retinoblastoma (Rb) tumor suppressor occurred in 78% of cases, alterations in the receptor tyrosine kinase/RAS/PI3K pathway were found in 88% of all GBMs and tumor suppressor p53 pathway was altered in 87% (*figure 1*) [15]. The following explanations of these three pathways are simplified; nevertheless, they give a short overview of the underlying pathogenesis.

Starting with cyclin-dependent kinase inhibitor 2A (cdkn2a) deletion, it should be mentioned that it is found in IDH-wildtype and -mutant GBMs [5]. Cdkn2a is a gene encoding for proteins that act as tumor suppressors by activating the Rb tumor suppressor protein family through inhibition of a Rb-inhibitor named cyclin-dependent kinase 4 and 6 (cdk4/6) [16]. Notably, reduction of RB1 expression is also caused by methylation of its promoter, which was mostly observed in IDH-mutant GBMs. Interestingly, this methylation could not be found in low-grade and anaplastic astrocytoma, leading to the conclusion that this process might be a late event in IDH-mutant GBM development [17].

Another important pathway is induced by epidermal growth factor receptor (EGFR) which is of great importance for IDH-wildtype GBMs [18]. Amplification of this receptor occurs in approximately 36% of IDH-wildtype GBMs, a significantly higher share than in IDH-mutant GBMs (8%). Also, EGFR overexpression is connected to GBM development, especially the most common specific mutation EGFRvIII. This mutation affects the receptor by deletion of exon 2-7 resulting in a shorter extracellular domain [14 18 19]. EGFR promotes the expression of oncogene PIK(3)K. As the PTEN (phosphatase and tensin homology) gene inhibits the pathway triggered by EGFR, it is obvious that mutations of this gene, which were found in 25% of GBMs, promote GBM development [14 20].

Finally, the p53 pathway has great relevance especially in IDH-mutant GBMs. Mutations in the p53 gene can be detected already in preliminary stages of GBM such as diffuse and anaplastic astrocytomas and lead to uncontrolled cell proliferation [17 18]. In this pathway murine double minute 2 (MDM2) is another relevant gene. It can be overexpressed and thereby inhibit p53 by forming a complex with the tumor suppressor [17].

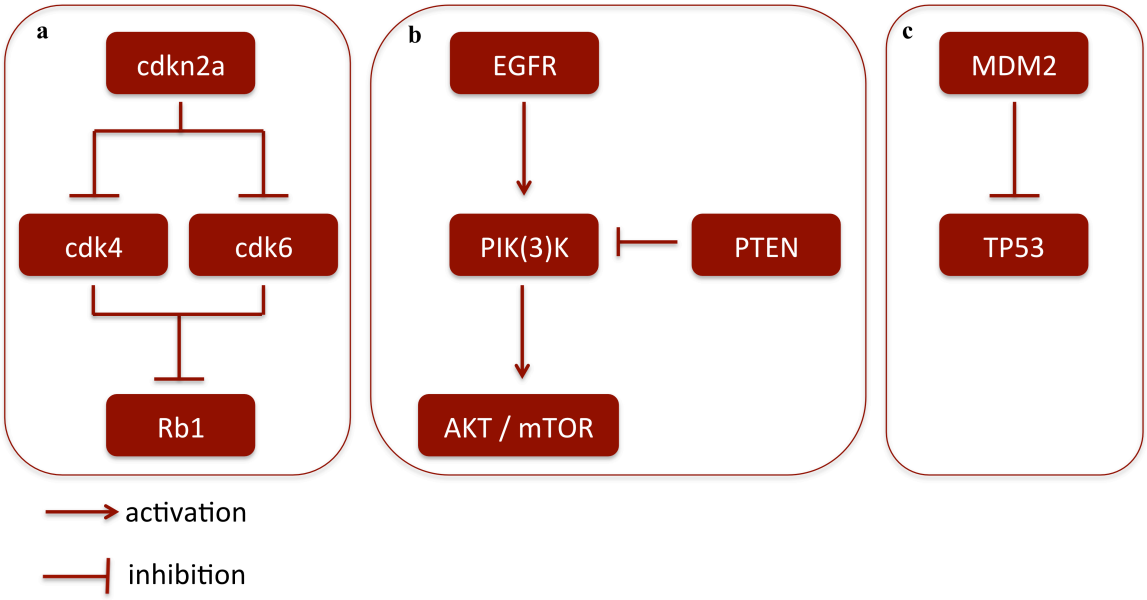

Figure 1.
Molecular drivers of GBM pathogenesis.
cdkn2a/Rb1 pathway: cdkn2a inhibits the expression of Rb1-inhibitors cdk4 and cdk6 which results in stronger expression of the tumor suppressor Rb1. Mutation of cdkn2a leads to lower expression of Rb1 (a). EGFR/PIK(3)K/AKT/mTOR pathway: amplification of EGFR leads to strong activation of oncogene PIK(3)K which promotes cell proliferation and inhibits apoptosis by activation of AKT/mTOR (b). p53 pathway: MDM2 inhibits p53 by forming a complex with the tumor suppressor (c).

Adapted from Brennan et al., Cell 2013.

1.1.5 Clinical presentation of the tumor

As primary GBM is a fast growing tumor, the patients present themselves with a short clinical history when being diagnosed. Studies have shown that more than 50% of the affected people had symptoms for only three to six month [21]. Moreover, secondary GBM developing from low-grade gliomas are accompanied by a clinical history of several years [21]. But as the majority develops and progresses fast, one of the biggest issues in clinical diagnostics is the demarcation to other neurological diseases such as stroke.

About half of the GBM patients suffer from headaches. As there is plenty differential diagnosis for this clinical symptom, a good anamneses focusing especially on the

development of pain is indispensable [22]. Headache symptoms become suspicious when occurring for the first time at an age older than 50 years, showing unilateral localization or progression in severity. Tumor associated headaches are mostly the result of an increased intracranial pressure which itself is a consequence of tumor growth and peritumoral edema [21]. Other signs of an increased intracranial pressure are nausea as well as papilledema, a swelling of the optic disc. Especially the latter symptom is rarely seen in our days due to earlier detection of the disease [21 22].

As stated above, necrotic areas in GBM are visible under the microscope. Focal symptoms depend on the localization of the tumor [21 22]. Tumors in the temporal lobe can lead to hearing and visual problems [21]. Furthermore, changes in personality can be observed when tumors are located in the frontal lobe and then have to be distinguished carefully from dementia and other psychiatric disorders [21]. In general, cognitive disorders are one of the main findings in GBM patients, especially when older than 51 years [23]. Finally, big tumors can also lead to incontinence and reduced sense of balance [22]. For younger patients, seizures with a focal onset are assumed to play an important role in diagnostics, as they may be the first clinical symptom of a GBM [21 22 23].

1.1.6 Tumor imaging

Patients suspected to have a brain tumor normally undergo brain magnetic resonance imaging (MRI) with and without contrast enhancement [12]. Computer tomography (CT) is used for patients with contraindications for MRI, for example when having a pacemaker implanted [22]. The superior soft tissue contrast of the MRI allows better visualization of the tumor and its heterogeneity than CT scans. On T1-weighted MR scans lesions occur hypointense whereas they are hyperintense in T2- and proton weighted images [12]. In MR scans enhanced with gadolinium, the necrosis – which is a hallmark of GBMs and required for a WHO grade IV diagnosis – can be seen in the center of the lesion, the tumor mass is irregularly shaped and a dense ring of gadolinium enhancement might be observed [24]. The necrotic area can be surrounded by edema [25]. Normally the tumor is unifocal but can also be multifocal in some cases [22]. In a CT scan the tumor appears as a hypodense area compared to

the brain tissue. Furthermore, a midline shift caused by lateralization resulting from edema may be observed [12]. In recent years, new imaging methods were developed or existing ones were improved. This now enables the evaluation of hemodynamic behavior, tissue architecture and cellular metabolism of gliomas [21]. One issue in diagnosing is the differentiation between tumor- and treatment-induced necrosis [12]. Therefore, single photon emission computer tomography (SPECT) and positron emission tomography (PET) are helpful diagnostic tools [12].

1.1.7 GBM therapy

A lot of effort has been made to improve GBM treatment [26]. Nevertheless, it remains one of the most challenging tasks in clinical oncology due to the heterogeneous and complex biology of the tumor [27]. Besides fighting the tumor, reducing its symptoms is a very important component of a holistic treatment. As stated above, increased intracranial pressure accompanied with headaches or nausea is a main issue. To reduce both symptoms, corticosteroids like dexamethasone (which is preferred for its low mineralocorticoid activity) are used at a dose of 12 to 16 mg/day [22]. Furthermore, antiepileptics are administered when seizures appear. Levetiracetam is one of the preferred drugs in this setting, as it has almost no drug-to-drug interaction with chemotherapeutics and a low toxicity profile. Notably, evidence for prophylactic use in GBM patients without seizures could not be found [28 29].

The current standard in GBM treatment includes – if possible – safely maximal surgical resection which is followed by radiation combined with temozolomide (TMZ) and an adjuvant chemotherapy with TMZ [1 30 31].

The main issue in surgery is the very invasive behavior of GBM, which makes a complete resection of the tumor quite difficult [32]. Additionally, a tumor located next to eloquent areas of the brain such as areas for speech and motor function control as well as human somatosensation could pose a challenge. As a result, infiltrating tumor cells usually remain in the tissue and lead to recurrence or disease progression [32 33]. Nevertheless, the extent of resection has prognostic value and can also diminish unpleasant symptoms like headaches, seizures and neurological deficits and

therefore improves the quality of life [34] [35]. Although prolonged through surgery, the median survival time – 15 months – remains poor [36].

The current radiotherapy dose used for radiation of GBM is 60 Gray (Gy) divided in 30 fractions. Fractionating the cumulative dose results in a reduction of harmful effects on non-tumor cells in the brain [37]. Radiation is used to kill remaining tumor cells, that were not eliminated by surgery [34].

Temozolomide (TMZ), an alkylating drug that is given orally, is the standard of care for GBM treatment [33]. Interestingly, it was shown that TMZ is less effective in patients with high levels of Methyl Guanine Methyl Transferase (MGMT) activity in tumor cells as MGMT works as a DNA repair protein. Consequently, tumor cells with active MGMT are protected from alkylating chemotherapeutics [38] [39] [40]. High methylation levels of the MGMT promoter gene result in lower MGMT activity correlating with a higher sensitivity of glioma cells for TMZ. Thus, MGMT promoter methylation serves as a prognostic factor for TMZ therapy [41]. There are other drugs such as carboplatin, oxaliplatin, etoposide and irinotecan, that are used in case of a missing response to the first line chemotherapy [21].

1.1.8 New experimental and targeted therapies

Recently, new targeted therapies such as the EGFR-antibodies Erlotinib and Gefitinib and others have been developed and are under clinical investigation, whereas targeted therapy with the anti-angiogenic VEGF-antibody Bevacizumab is already authorized in some countries for recurrent GBM [33]. So far, studies have shown no significant increase of survival under Bevacizumab therapy [42]. Unfortunately, several mechanisms limit the efficacy of current anti-angiogenic therapies. One of them seems to be the abnormal vasculature in GBM, which limits both the delivery of T-cells, antibodies and therapeutic drugs [43]. Recently, *Mastrella et al.* showed that targeting APLN/APLNR-signaling improves the efficiency of anti-angiogenic drugs like Bevacizumab and furthermore diminishes pro-invasive side effects of VEGFA/VEGFR2 blockers [44]. In agreement with these results, *Uribesalgo et al.* reported high APLN expression to be correlated with poor survival in advanced

breast and lung cancer in murine tumor models as well as in humans. They furthermore discovered that combination of APLN inhibition and anti-angiogenic therapy delays breast cancer growth in mice [45]. Both groups concluded that the APLN/APLNR pathway might be an important future target in GBM therapy.

1.2 APLN/APLNR-signaling and its role in diseases

1.2.1 Expression

Before focusing on the physiological and pathological functions of apelin, it is necessary to describe its structure and expression. Apelin is the product of posttranslational processing from a pre-proprotein, which consists of 77 amino acid residues and is processed from the C-terminal portion. The molecule occurs in different forms like apelin-12, the shortest version of apelin, apelin-13, -15, -16, -17, -19, -28, -31 and apelin-36, the longest active form and parental apelin peptide. Furthermore, there is a pyroglutamated form of apelin-13 ((Pyr1)-apelin-13) [46] [47]. It was shown that these apelin peptides have different receptor binding affinities and that shorter isoforms have a stronger activating effect on the apelin receptor (APLNR) than longer ones [48]. APLNR is a G protein-coupled receptor consisting of 380 amino acids and seven transmembrane domains. The receptor shows high sequence similarity to the angiotensin II receptor type 1. Nevertheless, angiotensin II cannot bind directly to APLNR [49] [50].

APLNR expression can be found in many different parts of the human body. It was detected in several regions of the CNS including the hypothalamus showing the highest expression [50] [52]. In the gastrointestinal tract it was discovered in pancreas, stomach, duodenum, jejunum and liver. Finally, APLNR can also be detected in kidneys and mammary glands [53] [54] [55] [56]. Looking at the expression on a cellular level APLNR is expressed in glial cells, cardiomyocytes, vascular and lung endothelial cells as well as smooth muscle cells [57]. In rats, highest APLN peptide concentrations have been found in the CNS, lungs, cardiomyocytes, pituitary and the mammary gland [58]. In mature breast milk APLN peptide levels can be increased by a factor of 7 to 20 compared to colostrum [59].

1.2.2 Physiological function

The following chapter concentrates on the function of the peptide starting with its effects on the brain.

Apelin increases water uptake into the ventricles when administered intracerebroventricular [60]. Depending on the dosage, it stimulates the secretion of adrenocorticotropic hormone (ACTH) and corticosterone hormone as well as the secretion of different hypothalamic-releasing hormones (CRH and vasopressin) [61]. Moreover, it decreases secretion of prolactin, thyroid-stimulating hormone (TSH) and growth hormone [60].

The effect of APLN on the cardiovascular system seems interesting as well. Research in rats has shown an immediate reduction of the blood pressure when injecting it intravenously [62]. APLN seems to have a protective influence on cardiac muscle cells and has relevance in angiogenesis, too [63] [64]. Intraperitoneal administration increases water uptake, similar to the aforementioned effect on intracerebroventricular injection [62].

1.2.3 Role of APLN in disease

APLN/APLNR-signaling is relevant in several diseases. In neurological disorders, it is necessary to mention the effect of APLN on pain modulation. APLN is discussed to be part of nociception, although, further investigation is needed to produce stronger evidence [65]. Inflammatory pain in rats seems to be correlated with decreased expression of endogenous APLN in the spinal cord, whereas normalization of apelin levels can reduce pain [66]. Apelin-13 furthermore improves the motor performance in mice, findings that match observations by *Kasai et al.* who have shown lower APLN expression levels to be correlated with progression of amyotrophic lateral sclerosis [67]. The APLN/APLNR system in the spinal cord is suspected to have a neuroprotective effect on this disease by protection from blood-brain barrier destruction [67].

As mentioned before, APLN has relevance in the context of hypertension. Exogenous APLN was shown to lower the blood pressure [48]. Nevertheless, the extent of blood pressure reduction depends on the applied APLN peptide isoform. In this context, apelin-12 shows the strongest effect of all isoforms [48]. Interestingly, when injecting APLN directly into the medulla of rats suffering from hypertensive disease, an increase of blood pressure could be observed which led to the assumption that the effect of APLN depends on the route of its administration [68]. In the cardiovascular system, APLN can also have a vasodilative effect by promoting nitric oxide (NO) and blocking vasoconstrictive angiotensin-II [69 70]. In addition, APLN seems to promote endothelial cell proliferation and therefore, could be important in the regulation of blood vessel diameters during angiogenesis [71]. Furthermore, APLN plays a role in the expression of several adhesion molecules and chemokine variations in endothelial cells. This might contribute to endothelial injuries and aggravation of hypertensive diseases [72]. Finally, a meta-analysis has shown that low circulating APLN levels are a possible reason for hypertension [73].

Metabolic disorders are another field in which the APLN/APLNR system might play an important role. A clinical study investigating healthy overweight men has shown improved insulin sensitivity after intravenous administration of (Pyr1)-apelin-13 [74]. In normal and obese insulin-resistant mice, intravenous administration of APLN at a physiological dose decreases glycaemia and enhances glucose utilization in the skeletal muscle as well as in adipose tissue [75]. It appears that APLN promotes glucose utilization and increases insulin sensitivity. These observations make the APLN/APLNR system a very interesting target for new therapies on type 2 diabetes and related metabolic disorders. Also, it might improve glycaemic control and endothelial disorders [76].

The APLN/APLNR system might be relevant in some respiratory and gastrointestinal diseases, too. During acute lung injury, an increase of APLN expression could be observed both in lung tissue and in plasma [77]. Similar observations were made in patients suffering from Morbus Crohn when analyzing mesenteric adipose and colon tissue [78]. In the context of both diseases, injection of apelin-13 decreases mucosal inflammation and improves the infiltration with inflammatory cells while diminishing

the expression of several pro-inflammatory cytokines [79]. Finally, blocking APLNR by using the antagonist apelin-F13A, an alanine substituted form of apelin-13 lacking phenylalanine, lowers mucosal blood flow, prostaglandin E2 production and vascular endothelial growth factor (VEGF) expression in rats [80]. In summary, there is strong evidence that APLN could play an important protective role against gastric diseases [81].

In mice suffering from hepatic diseases, injection of APLN decreases concentrations of transaminases and lactate dehydrogenase (LDH) in the serum [56]. Moreover, levels of APLN in patients suffering from chronic hepatitis C fluctuate and depend on the activity stage of the infection [82]. Some authors also suggest relevance for APLN/APLNR-signaling in liver fibrosis and cirrhosis [82]. Furthermore, protective effects of APLN on hepatic cells have been described in research. The mechanism behind this is an activation of the synthesis of vascular endothelial growth factor type A, platelet-derived growth factor-BB and cell survival [83]. The latter is achieved by reduction of cellular contents of triglycerides in human hepatoma cells and primary mouse hepatocytes, which leads to an inhibition of lipid accumulation [83 84].

The kidney is another organ that seems to be influenced by APLN/APLNR-signaling. Experiments on mice suffering from diabetes mellitus type I did not show an effect on body weight, glycaemia or blood pressure. Therefore, renal hypertrophy and albuminuria are reduced by APLN as well as glomerular enlargement and mesangial matrix deposition [85]. Finally, APLN reduces the expression of pro-inflammatory molecules in mice suffering from diabetes and can prevent cyclosporine-induced renal tubular injury [85]. Cyclosporine is mostly used for immunosuppression after organ transplantations [86].

APLN is considered to be relevant in the context of inflammation. The previously described mechanisms show that apelin has an influence on pro-inflammatory factor expression. Although, pro-inflammatory factors also influence apelin expression [87]. *Yang et al.* have shown macrophages to be influenced by the APLN peptide when it was added *in vitro*. It reduces the level of several cytokines such as monocyte

chemotactic protein 1 (MCP1), monocyte chemotactic protein 3 (MCP3), macrophage inflammatory protein 1 (MIP1α and MIP1β), vascular endothelial growth factor A (VEGFA), angiopoietin 2 (Ang2) and TNF-α in RAW264.7 macrophages [88]. This was also observed on primary rat peritoneal macrophages including a reductive effect of (Pyr1)-apelin-13 on phagocytosis and chemotaxis activity of the cells [89]. On the other hand, APLN can also act as a stimulator for inflammatory cytokine expression as shown by *Chen et al.* for the microglia cell line BV2, which I used in my experiments, too [90].

Finally, the role of APLN in malignant diseases is of special interest for my thesis. It was observed that APLN and APLNR are overexpressed in tumor tissues and induce intratumoral lymphangiogenesis and promote lymphatic metastasis [91 92]. An *in vivo* study on patients suffering from gastric diseases has shown higher APLN expression in the gastric cancer group than in the group suffering from chronic gastritis. Notably, serum APLN levels were approximately equal. Therefore, high tumor APLN levels were correlated with poor differentiation, lymph node and distant metastases in the gastric cancer group. In conclusion, it can be assumed that APLN promotes tumor invasiveness and influences metastases and tumor growth [93]. Colorectal cancer is another malignant disease that is influenced by APLN/APLNR-signaling. The anti-angiogenic monoclonal antibody Bevacizumab is used to treat this type of cancer [94]. Nevertheless, there is a major group of patients that does not respond to therapeutic strategies based on this drug [94]. Studies have shown that there is a correlation between non-responders and high endogenous APLN expression, which let to the assumption, apelin might act as a compensator of VEGF signaling loss caused by Bevacizumab [94]. In conclusion, APLN could be seen as a predictive biomarker for Bevacizumab response in patients with colorectal cancer [94]. Furthermore, APLN/APLNR-signaling also induces the morphological and functional maturation of blood vessels in tumors and improves immunotherapy by normalizing vessels [95]. APLN and APLNR overexpression was also observed in clinical glioblastoma samples. It was observed that apelin-13 and -36 increase the number of tumor spheres [96]. Finally, patients with non-small cell lung cancer and APLN overexpression show lower response to chemotherapy than patients with normal APLN levels [97]. Studies on another tumor type, called cholangiocarcinomas, have

confirmed the previously described effects of APLN. Also in this tumor APLN promoted tumor angiogenesis and proliferation. Inhibition of APLN by administration of an intravenous APLNR antagonist (called ML221) resulted in reduced growth of cholangiocarcinomas [98]. *Mastrella et al.* showed similar effects for GBM. They found reduced tumor angiogenesis when targeting APLN/APLNR-signaling [44].

1.3 Microglia and macrophages

1.3.1 The function of microglia and macrophages in the brain

Microglia was first mentioned by *Santiago Ramón y Cajal* in 1913 who described them as the "third element" of the central nervous system (CNS). They were named a few years later by *Pío del Río Hortega* [99] [100] and constitute 5 to 10% of all cells in a physiological brain [101]. Originally, microglia cells were thought to originate from the neuroectoderm, however it was recently shown that they arise from the embryonic yolk sac, where precursor cells also differentiate to macrophages for other tissues [102] [103] [104]. Interestingly, maintenance of the CNS microglia population in the adult depends much more on self-renewal than it is based on development from blood-circulating monocytes [105] [106]. Blood-associated myeloid cells are quite different from the precursor cells of microglia and most tissue macrophages in their developmental behavior and function [106] [107]. Furthermore, it was shown that microglia influence the brain more than previously assumed. As they interact with almost all components of the CNS, they take a key position in brain function and tissue integrity [108] [109]. They show several activation states and are able to adapt quickly to their environment in order to fulfill different functions [110] [111] [112]. They play a role in CNS development like neurogenesis [113] [114], in programmed cell death [115] [116] [117], in synapse elimination [118] [119] and in the remodeling of neural circuits [120] [121] [122] [123]. Due to this impact on brain homeostasis, it seems obvious that malfunction of microglia leads to several diseases and moreover is implicated in almost all injuries of the CNS [124] [125] [126].

Monocytes fulfill different functions that can be related to expression levels of different genes. The chemokine receptors CCR2 and CX3CR1 are used to describe subpopulations of macrophages and enable to distinguish between inflammatory monocytes (Ly-6C^{hi} CCR2^{+} CX3CR1^{lo}) that are recruited to inflamed tissue and

resident monocytes (Ly-6C^{lo} CCR2^{-} CX3CR1^{hi}) that can be found along blood vessels [127] [128]. Moreover, CD45 antibodies are used to distinguish resident microglia (CD45^{low}) and macrophages of hematopoietic origin (CD45^{high}) [129]. Recently, new markers for differentiation of microglia and macrophages have been described. *Bennett et al.* identified transmembrane protein 119 (Tmem119) to be a microglia-specific marker. In GBM, *Bowman et al.* found CD49d to be repressed in tumor associated microglia compared to macrophages [130] [131]. These markers help to distinguish between microglia and macrophages and therefore allow further studies of myeloid cell compositions in a certain environment [132]. Blood-borne macrophages are found in brain tissue especially in the context of brain cancers and neurodegenerative disorders [133].

During development of yolk sac derived macrophages, signaling via the colony-stimulating factor 1 receptor (CSF1R) features a very important role and is furthermore essential in the maintenance of microglia by influencing proliferation and differentiation as well as survival of macrophages [134] [102]. In adults, constant CSF1R signaling is necessary for microglia maintenance in the brain [135]. Besides CSF1R, there exist several other pathways with an impact on myeloid cell differentiation that are critically influenced by the brain environment [136] [137] [138]. Transforming growth factor-β (TGF-β) was shown to significantly influence the expression of microglial signature genes. It affects microglial specification and might have an anti-inflammatory and pro-survival impact as well [139] [140] [141]. Other factors of importance in microglial differentiation are the microglia-specific transcription factor SALL1 and the transmembrane protein negative regulator of reactive oxygen species (NRROS), that promote a quiescent microglial identity in the CNS environment [140].

As microglia was shown to affect different brain functions, they are able to scan the brain parenchyma and to contact different parts of the CNS with their processes [142] [143] [144]. Microglia is also a very important producer of complement factors in the brain. Complement is of relevance for the immune system as it targets bacteria and other microorganisms, but it also contributes to pruning of synapses, which has significance during brain development in young age [118] [119]. Reactivation of these pruning processes in adults might contribute to the pathogenesis of several

neurological and psychiatric disorders like Alzheimer disease [145] or schizophrenia [146]. Other diseases as well as cognitive ageing go along with synapse loss that is promoted by microglia produced complement [147]. Damaged or dead cells are eliminated by microglia phagocytosis [112], a process that is also used to control the size of the neuronal pool during neurogenesis [113] [148]. Furthermore, microglia are needed in the development and maintenance of the oligodendrocyte progenitor pool and the process of myelination of neurons [149]. Interestingly, microglia contribute not only to the development but also to the phagocytosis of myelin [150]. The last function of microglia and macrophages requiring attention for this research is their influence on endothelial cells. It could be observed that regulated by endothelial growth factor, they mediate blood vessel fusion, as they seem to be vascular fusion cells. In the context of cancer, TAM promote tumor angiogenesis by helping to establish a tumor vasculature [151].

1.3.2 The role of tumor-associated myeloid cells in glioblastoma

In this study I investigate tumor-associated macrophages (TAM) in the context of glioblastoma especially focusing on the influence of glioma-derived apelin. For a better understanding of my results it is necessary to give a short overview of how macrophages and microglia, together termed as myeloid cells, behave in the context of gliomas.

It is established that the cellular environment of solid tumors does not only consist of tumor cells, but that many other physiological cells like fibroblasts, endothelial and immune cells contribute to the local milieu in which neoplastic cells develop, grow and start to infiltrate other regions [152]. In the brain, astrocytes and oligodendrocytes must be added to this list [153]. These cells produce several growth and survival factors, chemokines, extracellular matrix constituents and angiogenic molecules and therefore create the microenvironment needed by tumor cells [153]. In glioblastomas, the most common malignant brain tumors, myeloid cells like microglia and macrophages represent up to 30 to 50% of these non-tumor cells that produce important stromal factors for tumor progression [152] [154] [155]. In low-grade gliomas it is a lower percentage of about 15 to 30%, in a non-neoplastic brain 10 to 15% [152]. CD68

is a protein that is highly expressed in monocytes. Interestingly, the more $CD68^+$ cells are found in gliomas, the higher their malignancy grade [156]. Furthermore, there seems to be an inverse correlation between $CD68^+$ myeloid cell density in gliomas and survival of patients, at least for pilocytic astrocytoma, a WHO 1 low grade glioma [157]. Several preclinical studies have shown a reduced tumor proliferation when microglial function was silenced [158] [159].

In 2019, *Böttcher et al.* have shown differences in the phenotypic signature of human microglia and peripheral myeloid cells. Furthermore, they have also found different phenotypes of microglia depending on the brain region. It remains unclear whether this observation implicates a region-specific function of these cells. Differences affect expression levels of markers such like $P2Y_{12}$, CD64, TGF-β1 and others [160]. Phenotypic differences of microglia were not only observed depending on the brain region but also on the age of the cell. Microglia of embryonic, juvenile and adult mice correlated to different gene expression profiles in a study by *Masuda et al.* [161].

Focusing on macrophages and microglia in the environment of gliomas, the mechanism behind the attraction of TAM towards the tumor is of certain interest. Former research has shown different chemokines, ligands of complement receptors, neurotransmitters and ATP to be part of these signaling pathways [153]. Monocyte chemoattractant protein-1 (MCP-1 or CCL2) was the first factor to be identified [162], although latest research questions this finding and suggests MCP-3 (CCL7) to be more important for chemoattraction of TAM [163]. Another molecule used by glioma cells to attract TAM especially to hypoxic areas is SDF-1 (CXCL12) [164]. Furthermore, glial cell-derived neurotrophic factor (GDNF) is mentioned to be used by glioma cells to attract TAM as well as colony stimulating factor-1 (CSF-1) [165]. When treating mice with the CSF-1 receptor antagonist PLX3397, TAM density in the glioma microenvironment was significantly reduced and GBM invasion attenuated [166]. Another glioma-released molecule with chemoattractive activity for TAM is the granulocyte-macrophage colony-stimulating factor (GM-CSF (CSF2)) [167]. There is evidence for several other chemoattractants, too. In my thesis, I investigated weather glioma-derived apelin can also be part of this chemoattractant system used by the tumor to enrich the microenvironment with TAMs.

As the glioma microenvironment contains a high density of TAMs, a range of studies addressed the question how these cells might influence the tumor. There is evidence that they promote tumor growth and invasion. A study has shown glioma cell mobility *in vitro* to be increased threefold in the presence of microglia, whereas oligodendrocytes and endothelial cells promote glioma motility only weakly [168]. So far, there are different cytokines known that are used by TAMs in order to induce glioma proliferation or migration: One is the stress-inducible protein 1 (STI1) [169], another is epidermal growth factor (EGF) [166]. A very interesting observation is the influence of CSF-1 that triggers a pro-tumorigenic phenotype of microglia and is released by the tumor cells. Using this mechanism, gliomas promote their own growth via microglial attraction and stimulation to tumor-supportiveness [170]. CCL2, already mentioned as a TAM-attractant molecule secreted by glioma cells, binds its receptor CCR2 and triggers IL-6 release in microglia which itself promotes glioma invasiveness [171]. Concerning invasiveness, TAM-derived transforming growth factor-β (TGF-β) has a similar effect on glioma cells as IL-6. It uses increased integrin expression and function as well as stronger expression of matrix metalloprotease-2 (MMP2) [172]. MMP2 needs cleavage via MT1-MMP before getting active. MT1-MMP is activated when being exposed to glioma cells, therefore, only in this microenvironment TGF-β-induced expression of MMP2 leads to a pro-tumorigenic effect of the metalloprotease [173]. To add further complexity, TLR2 is the receptor for MT1-MMP upregulation. Antibodies that block TLR2 can reduce glioma-induced MT1-MMP expression that leads to less MMP2 activity and finally results in attenuated glioma growth [174].

It is obvious that the mentioned signaling pathways are interesting as they represent possible targets for pharmacological glioma therapies. Due to its various functions in signaling, CSF-1 is currently one of the most investigated molecules [153]. Also minocycline, an antibiotic, is discussed as a helpful drug in glioma therapy as it interferes with microglia activation [153]. Nevertheless, it is still unclear, weather all glioma types interact with TAMs in the same way or if there are differences depending for example on the malignancy or molecular structure of the glioma subtype [153].

1.4 Reduction of APLN expression in U87 cells leads to reduced accumulation of Iba1-positive myeloid cells in orthotopically implanted xenografts

In former investigations of APLN and its role in glioma progression, the research group of *Dr. Roland Kälin* used U87 glioma cell xenografts in different mouse models. Using immunohistochemistry, myeloid cells were stained with an antibody targeting ionized calcium-binding adapter molecule 1 (Iba1) [175]. It was found that knockdown (KD) of APLN in U87 cells in a wildtype (WT) mouse leads to a significant decrease of the Iba1-covered area (*figure 2, a2)*. Interestingly, investigating xenografts with $U87^{WT}$ cells in an APLN-knockout (KO) mouse, the group found even higher density of TAM (*figure 2, a3*). $APLN^{KD}$ U87 glioma cells implanted in an $APLN^{KO}$ mouse showed a low myeloid cell density instead (*figure 2, a4*). The observation of Iba1-positive cells to be present at even higher cell density when APLN was missing in the host but expressed in the tumor cells led to the assumption that tumor-derived APLN could be responsible for myeloid cell attraction to the glioblastoma environment.

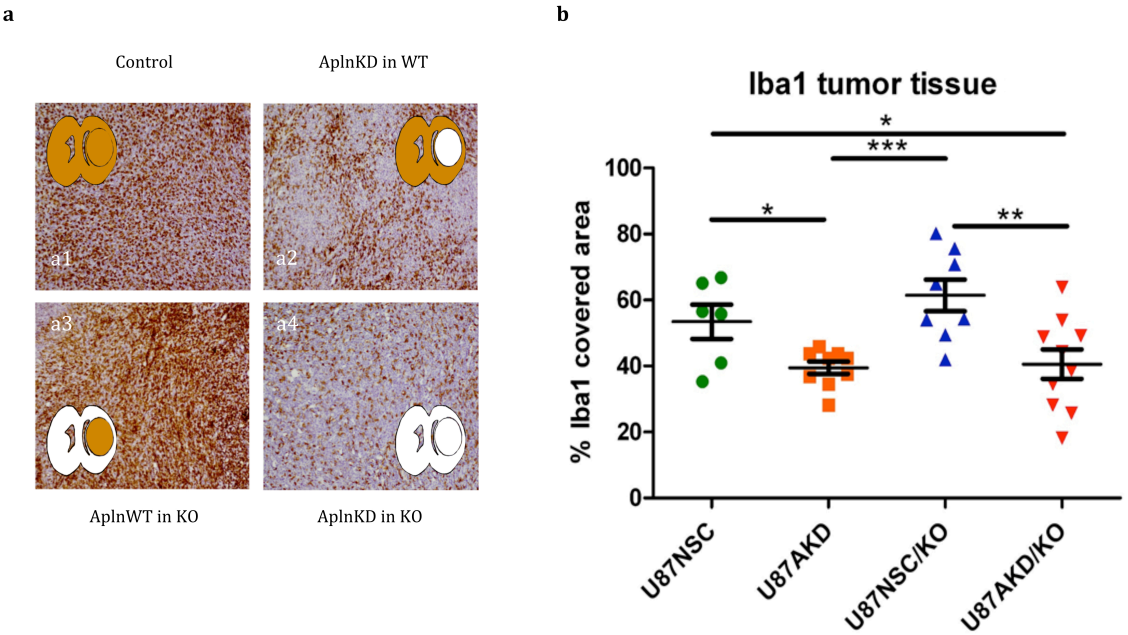

Figure 2.
Knockdown of APLN expression in U87 cells reduces the density of Iba1-positive myeloid cells. Invasion of Iba1-positive myeloid cells in different mouse models (a1-a4). Control with apelin-producing U87 glioma cells in a WT mouse (n=6) (a1). Apelin knockdown U87 glioma cells in a WT mouse (n=9) (a2). Apelin-producing U87 glioma cells in a KO mouse (n=8) (a3). Apelin knockdown U87 glioma cells in a

KO mouse (n=10) (a4). Quantification of the Iba1 covered area in different mouse models (b). *, $P < 0.05$, **, $P < 0.05$, ***, $P < 0.005$ (one-way ANOVA test). Unpublished data by *Kälin et al*.

2. Objectives of the thesis

Although coordinated strategies of chemotherapy, surgery and radiotherapy are used for treatment, glioblastoma remain among the most lethal cancers. New treatments must be designed to improve the survival of patients. Myeloid cells are known to be part of the microenvironment of gliomas and to enhance tumor angiogenesis and invasion. Therefore, these cells might be potential targets for future therapies against GBM.

Unpublished data from *in vivo* experiments by *Dr. Roland Kälin* using U87 glioma cell xenografts suggest a correlation between TAM-density and APLN-expression in GBM (*figure 2*) [176]. In the course of my thesis I wanted to investigate if there is a functional link between apelin expression in GBM cells and cell density of tumor associated myeloid cells.

Therefore the following three objectives were analyzed:

1. Does TAM density in newly established primary GBM models *in vivo* correlate with the expression levels of APLN in tumor cells quantified *in vitro*?

2. Does APLN have a direct impact on myeloid cells by acting as a chemoattractant?

3. Does APLN application have different effects on cultured microglia or macrophages?

For objective 1, qPCR was used to measure APLN expression levels in different glioma cell lines. Secondly, myeloid cells were stained in glioma cell xenografts using

Iba1. The area covered by TAM was measured under the microscope in different regions of the tumor.

For objective 2 and 3, the microglia cell line BV2 and macrophage cell line J774 A.1 were cultured. Both cells were tested for their migration behavior when being exposed to pure apelin-13 peptide or to conditioned medium of either APLN-expressing or APLN-deficient glioma cells.

3. Material and Methods

3.1 Material

3.1.1 Cell Culture

Material	Brand	Catalog number
Tissue Culture flask 25	TPP	90026
Tissue Culture flask 50	TPP	90076
Tissue Culture flask 150	TPP	90151
Dulbecco's MEM W 3,7 g/l $NaHCO_3$ W 1,0 g/l D-Glucose	Biochrom GmbH	FG 0415
PBS-Medium Buffer	Apotheke Klinikum der Universität München	APO-ST016
MEM NEAA (100x) Minimal Essential Medium	gibco® by Life Technologies	11140-050
FBS Superior	Biochrom GmbH	S 0615
Pen/Strep 10.000 Units/ml Penicillin 10.000 µg/ml Streptomycin	gibco® by Life Technologies	15140-122
EGF (Epidermal Growth Factor)	Biotechne	236-EG

FGF (Fibroblas Growth Factor)	PeproTech	100-18B
Trypsin/EDTA Solution (10x) In PBS	Biochrom GmbH	L 2153
Dimethylsulfoxide (DMSO)	Emsure® ACS Merck KGaA	317275
Trypan Blue Solution (0,4%)	Sigma-Aldrich®	T 8154
Bacillol® AF Surface Disinfectant	Hartmann	973380
Centrifuge Tube 15	TPP	91015
Falcon 50 ml Polypropylene Conical Tube	Corning Science	352070
CRYO.S™	greiner bio-one	126263
5 ml Stripette	Corning Incorporated	4487
10 ml Stripette	Corning Incorporated	4488
25ml Stripette	Cellstar® greiner bio-one	760180
Counting Chamber Neubauer	Paul Marienfeld GmbH&Co.KG	0640110

Heraeus Multifuge 1S Centrifuge	Thermo Scientific	75004311

Table 1: Material used for cell culture

3.1.2 RNA-Isolation

Material	Brand	Order number
Centrifuge 5415R	Eppendorf AG	542608357
Bio Photometer	Eppendorf AG	613120751
Thermomixer comfort	Eppendorf AG	
PBS-Medium Buffer	Apotheke Klinikum der Universität München	APO-ST016
Trypsin/EDTA Solution (10x) In PBS	Biochrom GmbH	L 2153
Centrifuge Tube 15	TPP	91015
Safe-Lock Tubes 1,5 ml	Eppendorf AG	0030123.328
Bacillol® AF Surface Disinfectant	Hartmann	973380

RNase ZAP™	Sigma Life Science	R 2020
Nuclease-Free Water	Qiagen	1039498
TRIzol® Reagent	ambion® by Life Technologies	15596018
Chloroform, >99,8%, A.C.S.	Sigma Aldrich	319988
2-Propanol, >99,5%	Carl Roth GmbH	9866.6
Ethanol Rotipuran®, >99,8%	Carl Roth GmbH	9065.1

Table 2: Material used for RNA-Isolation

3.1.3 cDNA-Synthesis

Material	Brand	Order number
PCR SoftTubes, 0,2 ml	Biozym Scientific GmbH	711088
QuantiTect® Reverse Transcription Kit	Qiagen	205313
Mastercycler personal	Eppendorf AG	533200583

Table 3: Material used for cDNA-Synthesis

3.1.4 Real Time Quantitative PCR

Material	Brand	Order number
PCR SoftTubes, 0,2 ml	Biozym Scientific GmbH	711088
Nuclease-Free Water	Qiagen	1039498
TaqMan™ Gene Expression Master Mix	Applied Biosystems	4369016
Mm APLN	Applied Biosystems	Mm00443562_m1
Mm APLNR	Applied Biosystems	Mm00442191_s1
Mm GAPDH	Applied Biosystems	Mm99999915_g1
Frame Star® Fast Plate 96	4titude®	4ti-0910/C
Plate Sealers	R&D Systems	DY992

Table 4: Material used for qPCR

3.1.5 Migration Assay

Material	Brand	Order number
Transwell® Permeable Supports 6,5 mm Insert, 24 Well Plate 8,0 μm Polycarbonate Membrane	costar® Corning Incorporated	3422
Paraformaldehyde (PFA)	Sigma Aldrich	P 1648
Normal Donkey Serum	Jackson Immunoresearch	017-000-121
DAPI	Fluka	32670
WGA 594 Agglutinin wheat	Life Technologies	W 11262
Raucotupf®	Lohmann & Rauscher International	11970
Rotilabo®-syringe filters, PVDF Pore size 0,45 μm	Carl Roth GmbH	P 667.1
BD 10 ml Syringe Luer-Lok™ Tip	Becton Dickinson S.A.	300912
Pyroglutamylated Apelin-13	Bachem	H-4568
Axiovert 25 CFL	Zeiss	451210

Table 5: Material used for migration assay

3.1.6 Immunohistochemistry

Material	Brand	Order number
Paraformaldehyde	Sigma Aldrich	P 1648
Microscope Slides Superfrost Ultra Plus®	Thermo Scientific	J 3800 AMNZ
Cover glass, 24x50 mm	LLG Labware	9.160829
Alexa Fluor 594 Donkey anti rabbit	Dianova	711-585-152
Triton-X	Fluka	93418
Normal Donkey Serum	Jackson Immunoresearch	017-000-121
Anti Iba1 Rabbit	Wako	019-19741

Table 6: Material used for Immunohistochemistry

3.2 Methods

3.2.1 Cell Culture

BV2 cells derive from the brain of a ten days old female mouse isolated by *Walker* in 1994 [177]. J774 A.1 cells were isolated from mice in 1968 by *Ralph* and established as a murine macrophage cell line [178]. The murine microglia-derived cell line BV2 and the murine macrophage-derived myeloid cell line J774 A.1 were maintained both under semi-adherent conditions in Dulbecco's MEM medium with stabilized glutamine containing 10% of fetal bovine serum (FBS), 5% MEM non-essential amino acids and 5% penicillin-streptomycin antibiotics. The cells were cultured in a humidified incubator with 95% O_2 and 5% CO_2 at 37 °C. After two to three days, vitality was checked under the microscope, the old medium was aspirated and cells were washed with PBS. Then new medium was added to the flask. As soon as the cells formed a monolayer on the bottom of the flask, they were washed and split by using the enzyme trypsin EDTA at 37 °C. BV2 cells detached after two minutes of trypsin incubation at 95% O_2 and 5% CO_2 at 37 °C. Due to their stronger adherence, J774 A.1 cells needed a longer period of time to detach. The combination of five minutes incubation and flipping the flask afterwards appeared to be a good treatment for these cells. Adding warm and fresh culture medium inactivated trypsin. The mixture of cells, medium and trypsin was transferred to a tube and then centrifuged for five minutes at 400 rpm. The obtained cell pellet was diluted with 1 ml medium. One part was returned to the old flask, the other part transferred to a new one filled with fresh medium. In order to freeze the cells, the pellet was diluted in 500 µl antibiotic-free medium to which 500 µl freezing medium (40% DMEM containing 10% FBS but no antibiotics, 40% FBS and 20% DMSO) were added.

My colleagues (*Mastrella et al.* [44]) obtained GBM stem-like cells (GBM 13, GBM 14 and NCH644) from human glioblastoma biopsies at the Medical Faculty Heidelberg and the Charité Medical University of Berlin and maintained them under stem cell cultivation conditions in DMEM-F12 supplemented with 1× B27, 5% penicillin-streptomycin, 10 ng/ml epidermal growth factor and 10 ng/ml fibroblast growth factor. For a direct tumor cell visualization, they marked the GBM cells with green

fluorescent protein (GFP) [44]. To investigate the effect of apelin knockdown in GBM cells they created both APLN-knockdown (AKD) cells by silencing the APLN gene using lentiviral shRNAmir constructs and nonsilencing controls (NSC) [44]. I used these GBM NSC cells in my *in vivo* and my *in vitro* experiments. AKD-cells were used in the *in vitro* migration assays only.

3.2.2 RNA-Isolation and cDNA-Synthesis

The cells used for the isolation of RNA were obtained by using the previously described cell culture methods. After centrifuging the cells and discarding the supernatant, 10 ml PBS were added to the tube followed by another centrifugation. Finally, 1 ml PBS was used to re-suspend the cell-containing pellet and the solution was transferred to a 1,5 ml Eppendorf, which was centrifuged for five minutes at 4 °C and 0,4 rcf afterwards. Under RNase- and DNase- free working conditions (using RNase ZAP^{TM}), the supernatant was discarded again and 250 µl RNA-free water were given to the pellet. Then 750 µl of trizol and 200 µl of chloroform were added. After mixing, the solution was stored on ice for ten minutes. I went on performing centrifugation at 4 °C and 12 000 rpm for 15 minutes resulting in three different layers. The lowest layer was pink and contained trizol and chloroform, the white middle layer represented DNA, the upper one represented RNA (transparent) needed for the next process step. This layer was transferred to a new Eppendorf by using a pipette. At this step it was crucial to prevent the RNA and the DNA-containing middle layer from mixing. 500 µl of isopropanol were added to the new tube in order to bind and precipitate the RNA linked with centrifugation for ten minutes at 4 °C and 12 000 rpm. After removing the supernatant, 1 ml of ethanol (75%, prepared in RNase-free water) was added and vortexed, then centrifuged for five minutes at 7,6 rpm and 4 °C. Without the supernatant the pellet was dried for five minutes in the opened Eppendorf. In the final step an amount of 40 µl RNase-free water was added before mixing the sample and incubate it in a thermomixer for ten minutes at 58 °C. The isolated RNA was stored on ice. The amount of RNA was measured in a spectrophotometer (calibrated with an only-buffer-containing cuvette) by placing 95 µl of buffer (10 mM Tris HCP) and 5 µl of the RNA sample in a cuvette and determining

the absorption. The results were averaged over three measurements and used to calculate the volume of RNA solution, resulting in 1 μg RNA.

cDNA-synthesis was performed with the QuantiTect® Reverse Transcription Kit by Qiagen. 2 μl of gDNA Wipeout Buffer, 7x were mixed with the volume of RNA solution containing 1 μg RNA. Depending on this volume, RNase-free water was used to fill up the tube to a total volume of 14 μl. The tube was incubated in the mastercycler for two minutes at 42 °C and immediately placed on ice. Meanwhile, the reverse-transcription master mix was prepared by using 1 μl Quantiscript Reverse Transcriptase, 4 μl Quantiscript RT Buffer, 5x and 1 μl RT Primer Mix. Then, the incubated RNA mix was added to reach a total volume of 20 μl and then incubated for 15 minutes at 42 °C. As a last step it was incubated at 95 °C for three minutes before storing the generated cDNA on ice or chilling it down at -20 °C.

3.2.3 Real Time Quantitative PCR

With the obtained cDNA, quantitative PCR was performed using TaqMan™ Gene Expression Assays for the murine genes APLN, APLNR and GAPDH. Therefore, a 96-well plate was used and the cDNA was diluted with RNase-free water (1:10 for J774 A.1 and 1:40 for BV2 cells). The plate was put on ice and each well filled with 11 μl of a solution containing 10 μl *MasterMix* and 1 μl primer. Afterwards, 9 μl of diluted cDNA was added to each well. Each primer was used in three wells, which led to nine wells per cell line in total. The plate was covered with foil before starting the qPCR in a StepOnePlus™ instrument with the standard running method provided by StepOne Software v2.2.2, increasing the cycle numbers to 45. APLN and APLNR expression levels were calculated by the ΔCT method and normalized on the housekeeping gene GAPDH.

3.2.4 Migration Assay

Corning Transwell® Permeable Supports with an 8,0 μm polycarbonate membrane were used in a 24-well plate and plated with 100.000 cells per insert diluted in 500 μl FBS-free DMEM medium obtained by the previously described cell culture. The lower compartment was filled with 1 ml of either DMEM plus pyroglutamylated apelin-13 at concentrations of 100, 200 and 500 nM or the supernatant of different glioma cell lines (human U87, GBM14 or NCH644 cells). My colleague *Mastrella* cultured these glioma cells in DMEM-F12 supplemented with 1X B27, 5% penicillin-streptomycin, 10 ng/ml EGF, and 10 ng/ml FGF. The supernatant was used directly for my migration experiments without any freezing. After 24 hours of incubation in a humidified incubator with 95% O_2 and 5% CO_2 at 37 °C, fluids and stationary cells on the upper compartment of the membrane were removed according to the manufacturer's instructions using a cotton swab. Subsequent to treating the migrated cells on the lower side of the membrane with 400 μl PBS (five minutes) two times, cells were fixed by using 400 μl pure PFA and washed again three times for five minutes in PBS. Then, cells were blocked for one hour at room temperature (blocking solution containing PBS with 5% donkey serum and 0,3% Triton-X 100). Afterwards, the nuclei were stained in 400 μl of a solution containing DAPI and PBS in a relation of 1:10.000 for two hours followed by staining of the cell membranes using wheat germ agglutinin (WGA 594) in PBS (1:200) protected from the light for one hour. As a final step, the amount of migrated cells was counted under the microscope.

3.2.5 Immunohistochemistry

Brain sections have been received from my colleagues. Their animal experiments had been approved by the local animal care committee of the Government of Oberbayern and had been conducted following animal welfare regulations (including 3R principle) of the National Guidelines for Animal Protection. The brain sections were washed in 500 μl PBS (three times for five minutes), blocked for one hour at room temperature in 500 μl PBS containing 5% donkey serum and 0,3% Triton-X and then incubated for 24 hours at 4 °C using Wako® rabbit anti-Iba1 primary antibody

diluted in the blocking solution with a relation of 1:500. On the second day, slices were washed again in PBS and incubated for two hours at room temperature with Alexa Fluor® donkey anti-rabbit 594 secondary antibodies in a dilution of 1:500 and To-Pro nuclear staining diluted 1:1.000. All antibodies were diluted in blocking solution (PBS with 5% donkey serum and 0,3% Triton-X 100). After washing the slices again with PBS, they were mounted with Dako Fluorescent Mounting Medium®. Pictures for Iba1-positive cells were taken in the center of the tumor, the invading zone, the peritumoral region and the periphery with a Zeiss microscope at 40x magnification. To quantify the amount of Iba1-positive cells, the area covered by Iba1-positive cells was measured in each picture and the average of three pictures was taken for each region. Therefore, I used ImageJ Software's color threshold method.

3.2.6 Statistical analysis

All statistical analyses were conducted using GraphPad Prism 6. Each variable was summarized in its distribution by using the mean standard variation (SD). The number of replicates, individuals and repetition of independent experiments are described in the text of each figure. In experiments with just two different conditions or populations, statistical analysis was performed by using an unpaired student's t-test and the values are reported as the mean ± SD. When more than two groups were compared, a one-way ANOVA with Newman-Keuls *post hoc* test was used for determination of statistical significance. Values were again reported as the mean ± SD.

P values are indicated as *, P<0,05; **, P<0,005; ***, P<0,0005; ****, P<0,0001 in all results.

4. Results

4.1 APLN-expression levels in glioma cells *in vitro* correlate with TAM density *in vivo*

As shown in *figure 2*, manipulation of APLN expression in U87 glioma cells changed the density of TAM within the tumor. Therefore, I asked if high tumor APLN expression could be connected to high density of TAM and low APLN expression to low TAM density. For this thesis, patient-derived GBM xenograft models (GBM13, NCH644, GBM14) were used to further investigate the correlation between the APLN expression of gliomas and TAM density. These xenografts recapitulate the pathological parameters of human GBM better than U87 xenografts, as they show typical histopathological signs such like high vascularization and intratumoral necrosis. In addition, I used a newly developed mouse model established by *Mastrella et al.* that carries the typical tumor drivers EGFRvIII and cdkn2a^{KO} [44]. Iba1-staining was used to make TAMs visible.

Figure 3 shows the APLN expression levels of the previously mentioned glioma cells. Data was generated and partly published in 2019 by *Mastrella et al*. I adopted their data for this thesis and correlated it to my own results. *Mastrella et al.* detected the weakest expression for GBM13 cells followed by NCH644, whereas GBM14 and mouse-derived cdkn2a^{KO}EGFRvIII glioma cells were related to higher APLN expression.

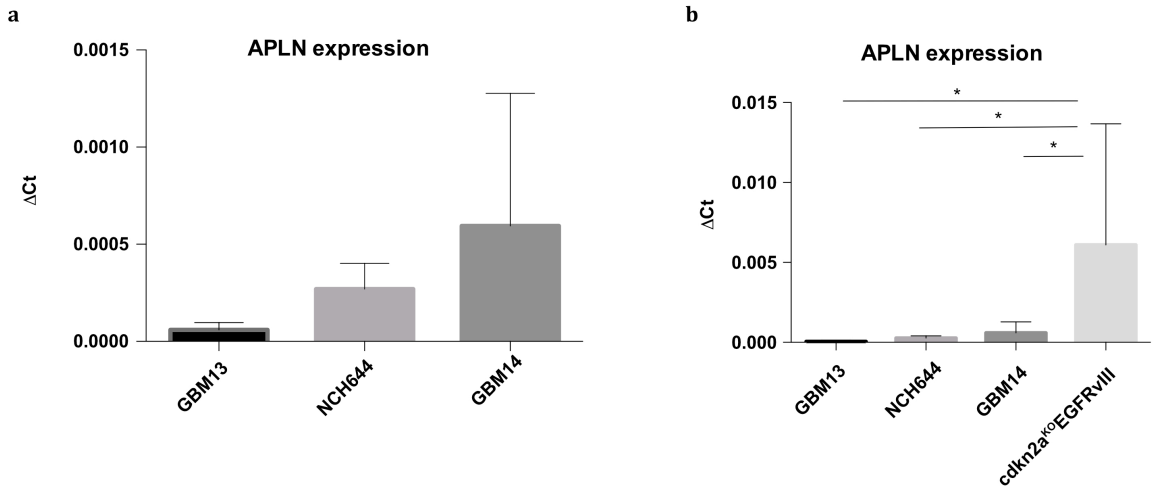

Figure 3.
APLN expression levels in different human and murine glioma cell lines (a-b). APLN expression levels of human glioma cell lines GBM14 (sample size N=7), NCH644 (N=4) and GBM13 (N=3) differ. GBM14 is related to the highest expression levels, GBM13 to the lowest, although results are not significant (a). Compared to the murine glioma cell line cdkn2a^{KO}EGFRvIII (N=4), APLN expression levels of all human glioma cells are significantly lower (b); statistical significance (two-tailed student's t-test) is indicated. *, P<0,05. Adopted from *Mastrella et al.* 2019.

To test if these APLN expression levels correspond directly with the amount of Iba1-positive TAM, brain tumor tissue of different GBM mouse models was analyzed by immunohistology. Therefore, the new mouse models with orthotopic implantation of different glioma cells generated by *Mastrella et al.* were used [44].

When focusing on TAM density, four tumor and brain regions were suggested to be of special interest. The tumor center, as it is connected to strong angiogenesis, the invading zone where GBM invasiveness can be observed, the peritumoral but non invasive zone and the contralateral hemisphere as a control region where no influence of the tumor would be estimated (*figure 4*) [44].

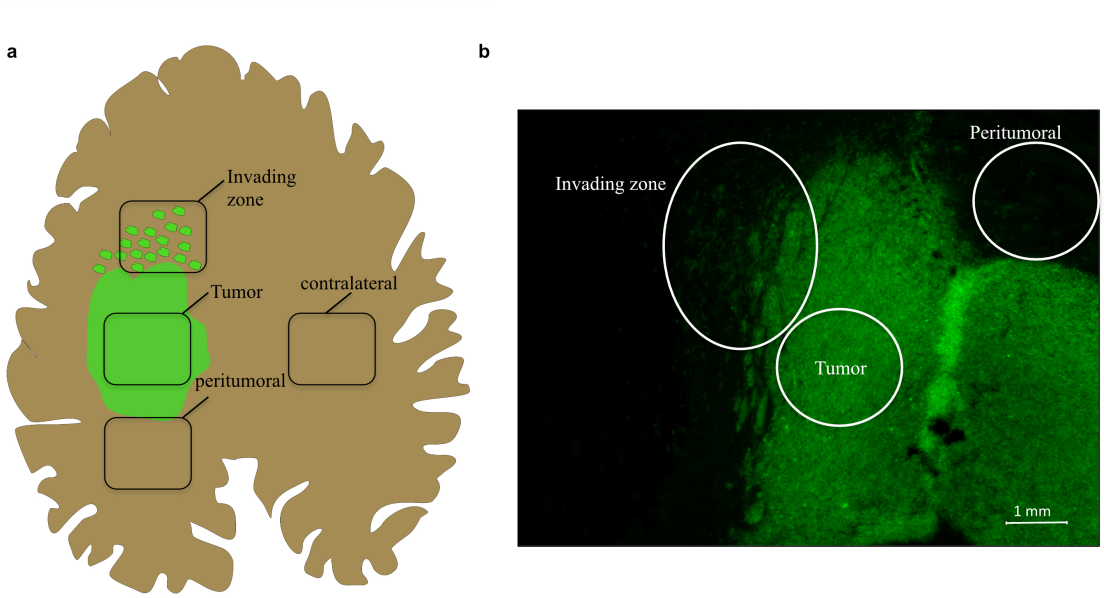

Figure 4
Definition of four relevant tumor-associated brain regions. Demonstrated schematic (a) and as fluorescence micrograph of a Cdkn2a glioma-cell injected mouse model (b). Cdkn2a tumor cells are shown in green. Tumor center (strong angiogenesis), invading zone (GBM invasion), peritumoral (non-invasive tumor border) and contralateral region are indicated. Scale bar = 1 mm.

For the tumor center, GBM13 injected mouse brains were found to show the lowest density of Iba1-positive cells. NCH644 and GBM14 treated mice had a higher amount of TAM compared to GBM13. The largest area covered by Iba1-positive cells could be observed in mouse brains injected with cdkn2a^{KO}EGFRvIII glioma cells (*figure 5)*. Differences were highly significant for all tumor entities when compared to cdkn2a^{KO}EGFRvIII. Significance was also found for GBM13 compared to GBM14 cells. Concerning the center of the tumor, my data shows that strong APLN expression of tumor cells is correlated to high myeloid cell density. Thus, the data shows a direct correlation.

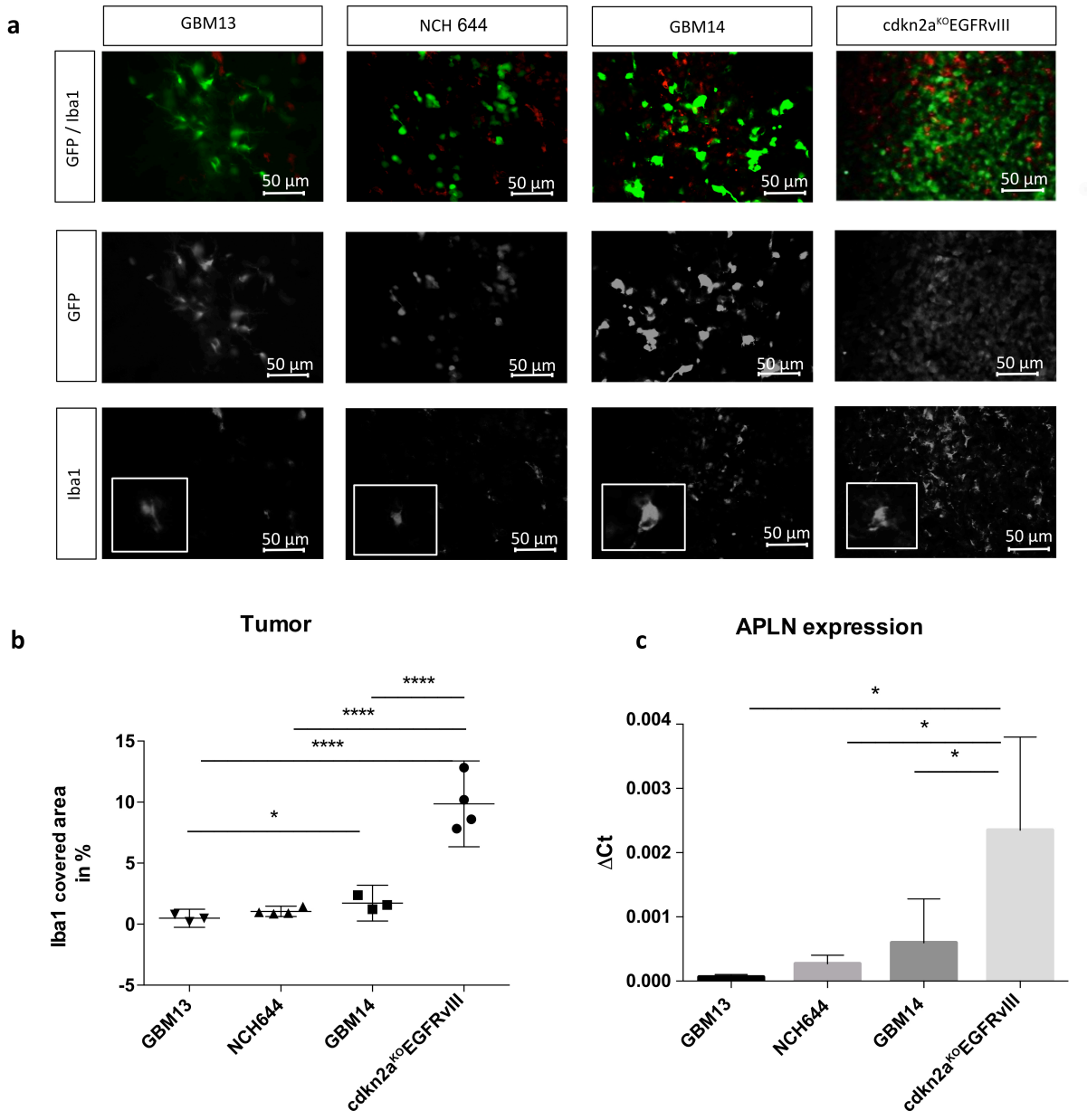

Figure 5
TAM density in the tumor region is directly correlated with the APLN expression level of tumor cells. GFP-stained tumor cells are shown in green (top row) and grey (second row), Iba1-positive myeloid cells in red (top row) and for a better contrast in grey (bottom row). Close-ups show a single, activated myeloid cell in the tumor region (a). Tumor region of mice injected with either GBM13 cells (sample size N=3), NCH644 cells (N=4), GBM14 cells (N=3) and cdkn2a^{KO}EGFRvIII (N=4) cells show significant differences in myeloid cell density (b). Simultaneously, APLN expression levels between these cells are significantly different (c). To facilitate direct comparison of APLN expression levels with TAM density, graph (c) is shown multiple times in figures 5-8. 40x magnification; statistical significance (one-way ANOVA plus *post hoc* test) is indicated. ****, P<0,0001; *, P<0,05; scale bar = 50 µm.

I demonstrated a direct correlation of glioma cell APLN expression and density of TAM for the tumor center. I continued my research focusing on additional brain regions, as the question remained whether the previously described correlation is a tumor center phenomenon only.

TAM have been reported to support tumor invasion, so I investigated their relevance in the invading zone as part of the GBM microenvironment [179]. NCH644 tumors were found to be very compact showing a minor degree of tumor invasion. For this reason, analysis of the invading zone was not possible. Nevertheless, TAM density was significantly different between cdkn2a^{KO}EGFRvIII samples, related to the highest myeloid cell density in the invading zone among all tested GBM entities, and GBM14 (*figure 6*). Again, this correlation reinforced the hypothesis of GBM APLN expression to be directly correlated with the density of TAM.

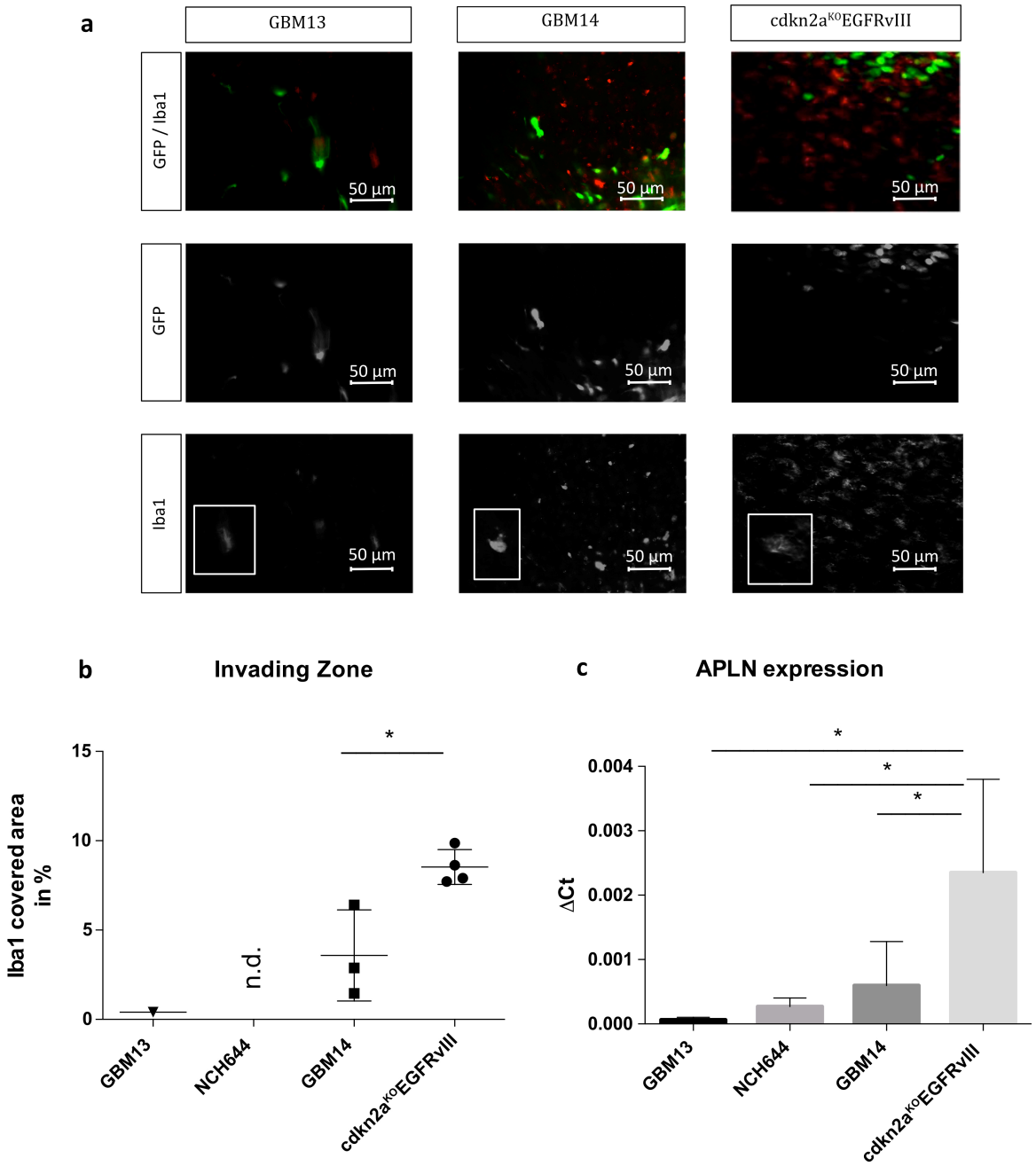

Figure 6

TAM density in the invading zone is directly correlated with the APLN expression level of tumor cells. GFP-stained tumor cells are shown in green (top row) and grey (second row), Iba1-positive myeloid cells in red (top row) and for a better contrast in grey (bottom row). Close-ups show a single, activated myeloid cell in the tumor region (a). Glioma-invading zone of mice injected with either GBM13 cells (sample size N=1), GBM14 cells (N=3) and cdkn2a^{KO}EGFRvIII (N=4) cells show significant differences in myeloid cell density. No invading zone could be detected for NCH644 tumors (n.d. = not detectable) (b). Simultaneously, APLN expression levels between these cells are significantly different (c). To facilitate direct comparison of

APLN expression levels with TAM density, graph (c) is shown multiple times in figures 5-8. 40x magnification; statistical significance (two-tailed student's t-test) is indicated. *, P<0,05; scale bar = 50 µm.

Additionally, two other tumor and brain regions were defined: the peritumoral region next to the glioblastoma not showing any signs of tumor invasion (*figure 7*) and the contralateral hemisphere (*figure 8*). As tumor cells secret APLN and have influence on GBM activities like angiogenesis or glioma cell invasion it remained unclear how far they influence myeloid cells. I suggested glioma-derived APLN to mostly influence the microenvironment of the tumor, especially the tumor center and the invading zone. As the contralateral hemisphere shows the largest distance from the glioma it was expected to be least influenced by GBM cells. Therefore, myeloid cell density should be similar in all mouse models in this region. Interestingly, the direct correlation of Iba1-covered area and APLN expression levels of the glioma could be found for all regions, which supported the hypothesis of APLN expression being directly correlated to myeloid cell density on the one hand. On the other hand, it was surprising to observe that gliomas might influence not only their microenvironment but also myeloid cells in the whole brain, too. A significant difference was detected for myeloid cell density as well in the peritumoral region as in the contralateral hemisphere when comparing cdkn2a^{KO}EGFRvIII tumors with GBM14 and NCH644 tumors. Also, GBM14 and NCH644 tumors differ significantly in their myeloid cell-covered area around the tumor and in the contralateral hemisphere. But nevertheless, they still show a direct correlation of APLN expression levels and TAM density.

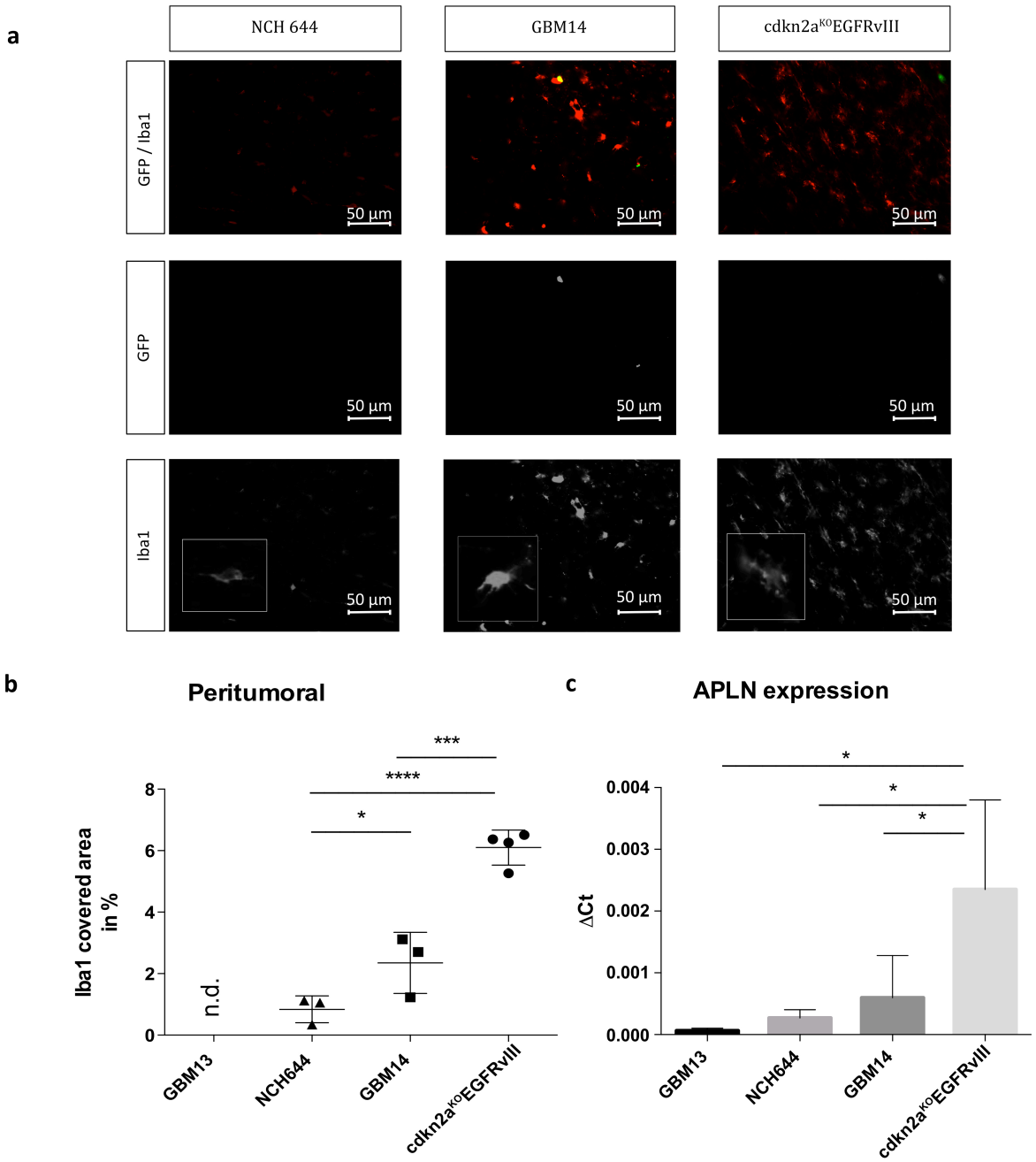

Figure 7
TAM density in the peritumoral region is directly correlated with the APLN expression level of tumor cells. GFP-stained tumor cells are shown in green (top row) and grey (second row), Iba1-positive myeloid cells in red (top row) and for a better contrast in grey (bottom row). Close-ups show a single, activated myeloid cell in the tumor region (a). Peritumoral region of mice injected with either NCH644 cells (sample size N=3), GBM14 cells (N=3) and cdkn2a^{KO}EGFRvIII (N=4) cells show significant differences in myeloid cell density. No peritumoral region could be detected for GBM13 tumors (n.d. = not detectable) (b). Simultaneously, APLN expression levels between these cells are significantly different (c). To facilitate direct comparison of APLN expression levels with TAM density, graph (c) is shown multiple times in figures 5-8. 40x magnification; statistical significance (one-way ANOVA plus

post hoc test) is indicated. *, P<0,05, ***, P<0,0005, ****, P<0,0001; scale bar = 50 µm.

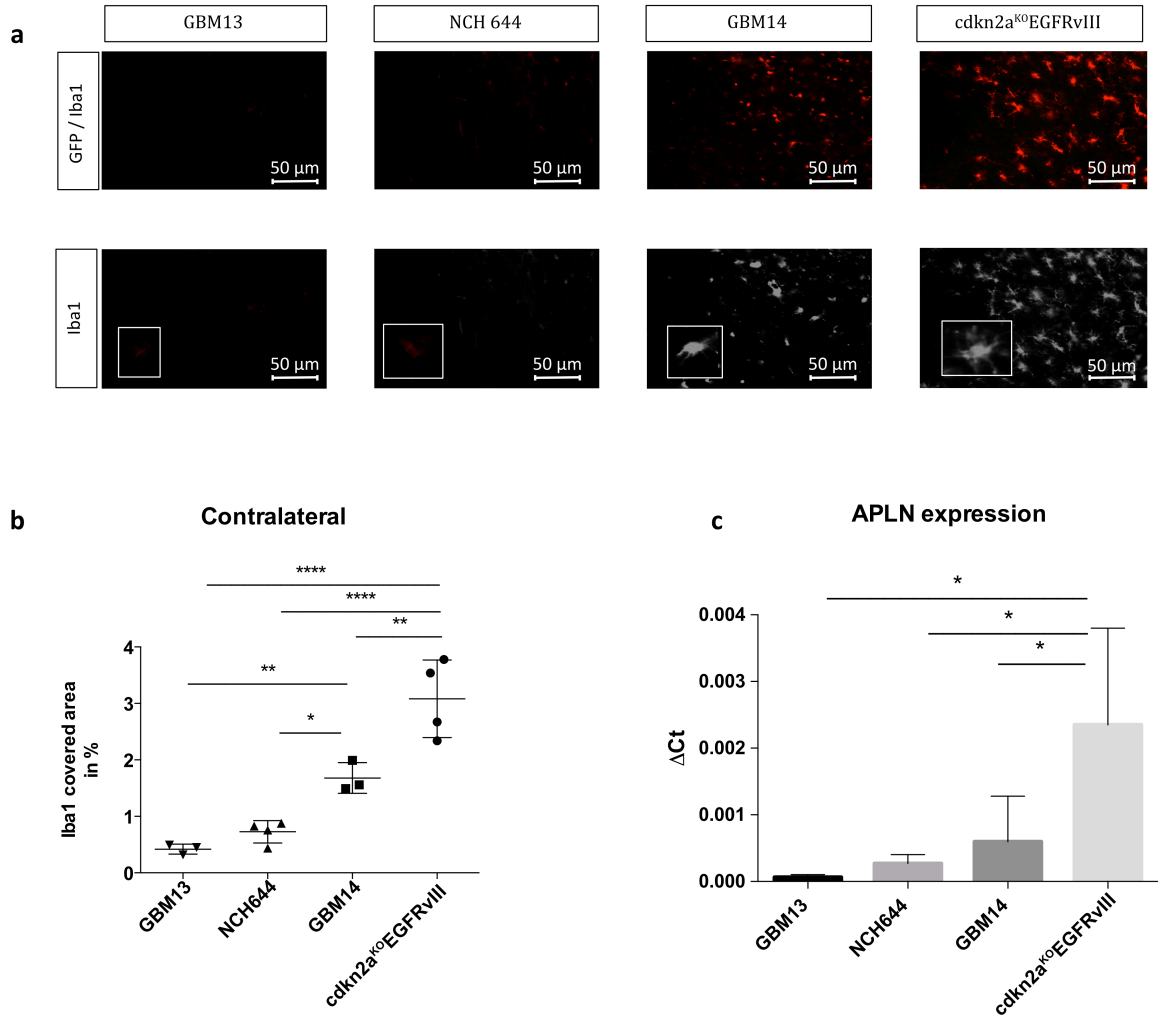

Figure 8
TAM density in the contralateral hemisphere is directly correlated with the APLN expression level of tumor cells. GFP-stained tumor cells are shown in green (top row), Iba1-positive myeloid cells in red (top row) and for a better contrast in grey (bottom row). Close-ups show a single, activated myeloid cell in the tumor region (a). Contralateral region of mice injected with either GBM13 cells (sample size N=3), NCH644 cells (N=4), GBM14 cells (N=3) and cdkn2a^{KO}EGFRvIII (N=4) cells show significant differences in myeloid cell density (b). Simultaneously, APLN expression levels between these cells are significantly different (c). To facilitate direct comparison of APLN expression levels with TAM density, graph (c) is shown multiple times in figures 5-8. 40x magnification; statistical significance (one-way ANOVA plus *post hoc* test) is indicated. *, P<0,05; **, P<0.005; ****, P<0,0001; scale bar = 50 µm.

Summarizing the findings of my *in vivo* experiments on myeloid cell density in brain samples of mice suffering from different glioma subtypes, the amount of APLN expression directly correlates with the area covered by Iba1-positive cells in all regions, not only in the tumor center and the invading zone, which are part of the microenvironment of gliomas. The direct correlation was observed in the whole brain, the macroenvironment, too.

Finally, I analyzed differences of the area covered by Iba1 in each mouse model. I aimed to figure out, whether the amount of myeloid cells is significantly higher in and around the tumor or not. It was only cdkn2a^{KO}EGFRvIII glioma cell injected mice that showed significant differences in the Iba1-covered area between tumor, invading and peritumoral zone as well as the contralateral hemisphere (*figure 9*). For GBM14, NCH644 and GBM13 no difference was observed. As Iba1 is not TAM-specific, it is obvious that physiological microglia and monocyte-derived macrophages are stained, too [180]. For the contralateral region it might be physiologically activated microglia more than TAM that was detected. Nevertheless, this finding needs further investigation to find out whether and how microglia in the contralateral hemisphere is influenced by glioma-derived apelin.

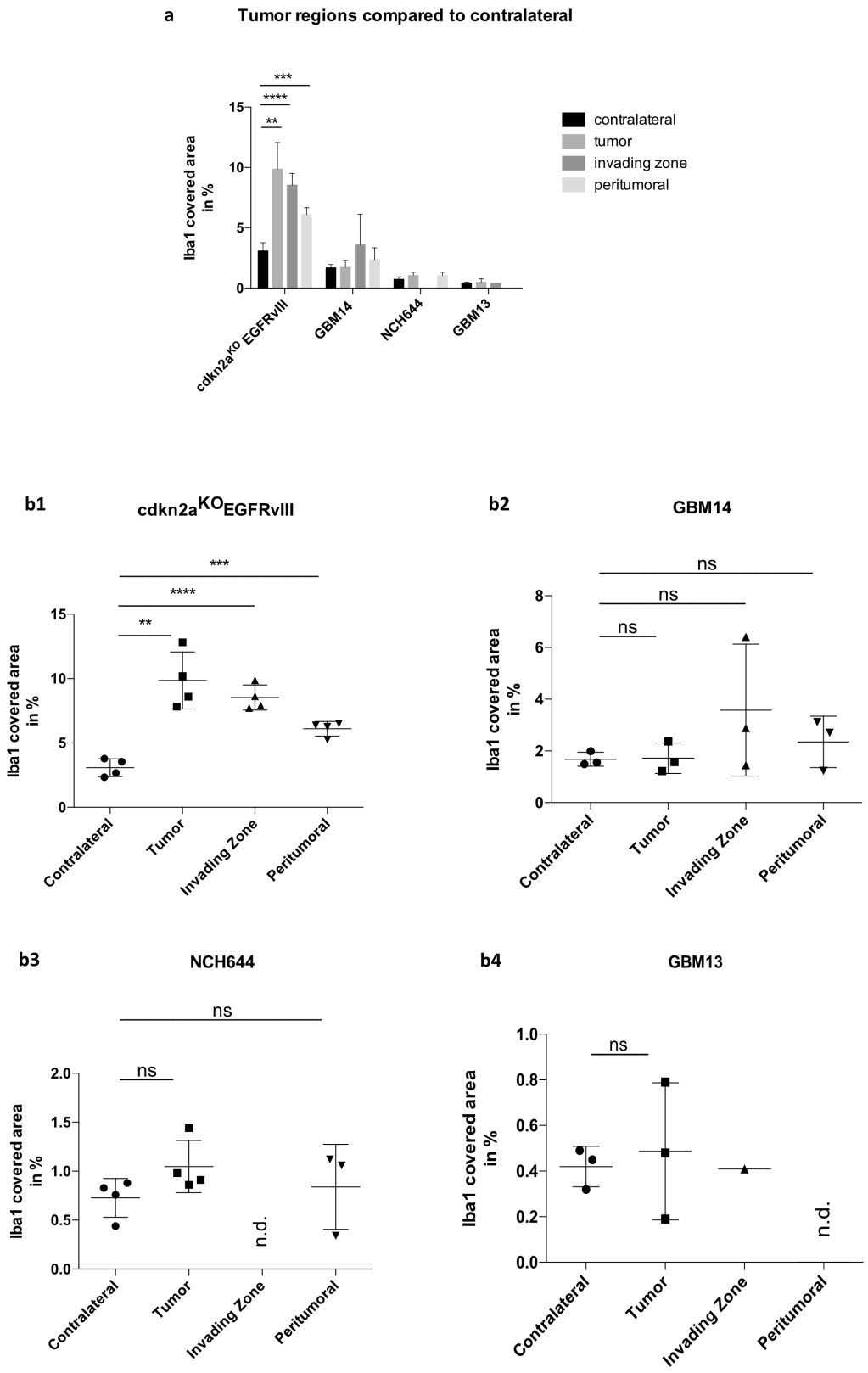

Figure 9
Except for brain samples of mice injected with cdkn2a^{KO}EGFRvIII glioma cells, the Iba1 covered area is not significantly higher in tumor-associated regions

than in the contralateral hemisphere (a-b). Only cdkn2a^{KO}EGFRvIII glioma cell injected mice showed a significantly higher concentration of Iba1 positive myeloid cells in regions associated with GBM (tumor center, invading and peritumoral zone) (a + b1). This was not seen in mice suffering from GBM14, (a + b2), NCH644 (a + b3) and GBM 13 (a + b4) glioma cell tumors. n.d. = not detectable. Statistical significance (two-tailed t-test) is indicated. **, P<0.005; ***, P<0,0005; ****, P<0,0001

4.2 APLNR is expressed in BV2 microglia and J774 macrophages

In the analysis of orthotopically implanted tumors I found that TAM density in different mouse models directly correlates with the APLN expression of glioma *in vivo*. To investigate if APLN can cause a cellular response on myeloid cells *in vitro*, I tested if APLN has an effect on cell migration. Therefore, two myeloid cell lines, BV2 microglia and J774 macrophages, were used. Both were shown to be part of the glioma microenvironment [181]. Nevertheless, few specific studies have been performed on differences and similarities of the migration of microglia and macrophages in the context of GBM so far. To investigate if BV2 and J774 cells have the potential to react on APLN, I analyzed if APLNR is expressed in these cells. Therefore RNA was isolated and reverse transcribed into cDNA, which was used to perform real time quantitative PCR.

The results showed APLNR to be expressed in both cell lines (*figure 10 a*). This is in line with unpublished results obtained from GBM xenografts by my colleague *G. Mastrella*, who showed APLNR on TAM by fluorescent immunostaining [182]. To see if APLN/APLNR-signaling is influenced by inflammatory signals, I checked the expression of APLNR after treatment with Lipopolysaccharid (LPS), which is known to act in an immune stimulating way [183]. Interestingly, APLNR expression in macrophages decreased – although not significantly – after stimulation. For microglial BV2 cells the receptor was not even detectable anymore (*figure 10 b*). This observation matches findings by *Leeper et al.* in 2009 and *Obara et al.* in 2014 and will be discussed later in my thesis [184] [185]. Importantly, the results obtained by qPCR allowed further investigation of the effect of apelin on BV2 and J774 cells as the

receptor for the peptide was found on both cells and therefore interaction of apelin and TAM was possible.

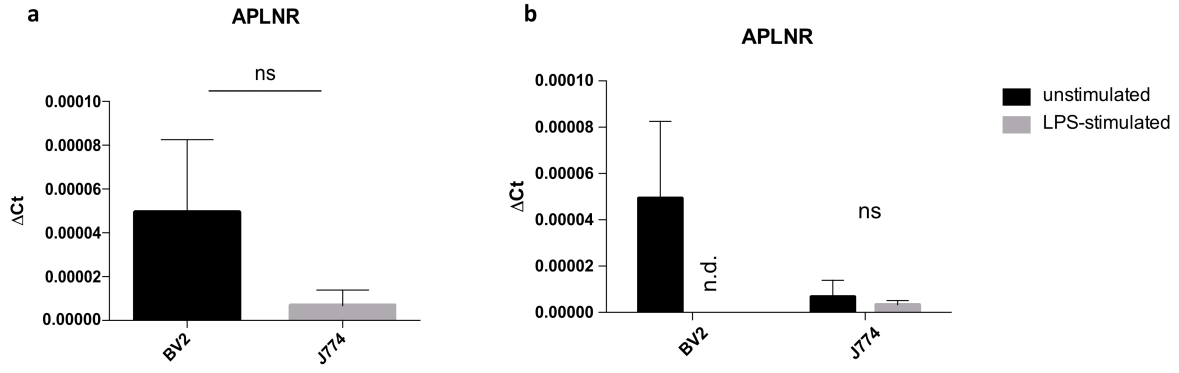

Figure 10
APLNR expression levels in BV2 microglia and J774 macrophages are not significantly different. After immunological stimulation they are reduced. Expression levels of APLNR show no significant differences in BV2 and J774 cells (a). Unstimulated BV2 microglia (number of replicates n=4) show stronger expression than LPS-stimulated BV2 cells (n=3), which show no APLNR-expression at all (b). Also J774 macrophages (n=3) show decreased expression when being stimulated with LPS (n=3), too (b); n.d. = not detectable. ns = no significance.

4.3 Migration assays investigating migratory behavior of BV2 and J774 cells

4.3.1 Fetal calf serum (FCS) has no effect on migratory behavior of myeloid cells

BV2 and J774 cells were cultured in a medium containing 10% fetal calf serum (FCS). As FCS contains many different growth factors and chemoattractants, the first aim was to test if this supplement affects BV2 and J774 migration behavior and thus to find the best experimental conditions. According to a protocol by *Justus et al.* from 2014, 100.000 cells were defined as the best number of migrating cells in the upper compartment [186]. This amount of cells was used and plated either in FCS-containing medium or FCS-free medium in the lower compartment of the migration assay. The upper compartment was filled with FCS-containing or FCS-free medium, too. There

were no significant differences being observed as the number of replicates was low, but a tendency to higher migration with FCS exposition (*figure 11*). I decided to investigate the role of APLN on myeloid cells under FCS-free conditions.

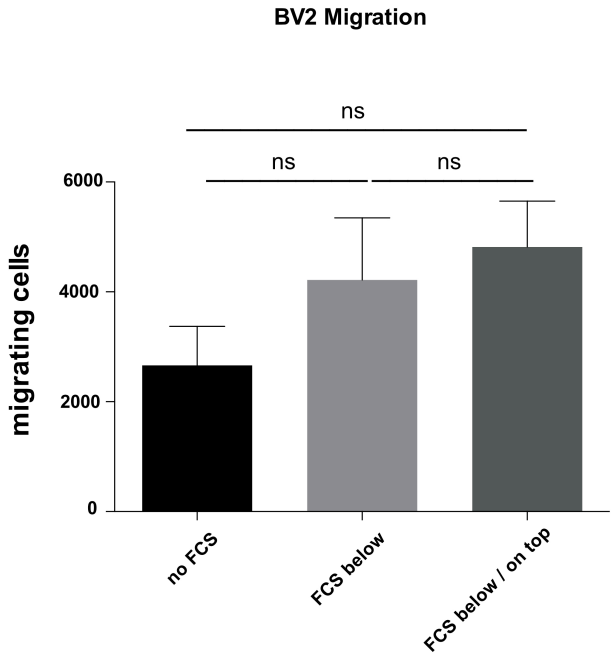

Figure 11
FCS as component of the culture medium has no influence on BV2 migration. The effect of FCS on BV2 migration was investigated for different conditions including FCS in the lower compartment only as well as in both compartments of the insert and was then compared to the control condition (no FCS). Number of replicates n=3, sample size N=1. No statistical significance (two-tailed student's t-test and one-way ANOVA plus *post hoc* test) could be found. ns = no significance.

4.3.2 Apelin-13 peptide increases BV2 migration

I have shown that BV2 and J774 cells express APLNR. This makes them responsive to APLN peptides. As APLN is a chemotactic factor, I performed migration assays using apelin-13 peptide. This isoform was previously reported to be the most potent chemoattractant to primary endothelial cells among the apelin peptides by *Kälin et al.* in 2007 [187]. To evaluate the optimal concentration of the peptide, a dose-escalation

experiment was performend to determine the concentration provocing the strongest possible migration of myeloid cells. A setup with just DMEM (without FCS) served as a control. Under all conditions I could show that BV2 microglia is attracted by apelin-13. For a peptide concentration of 100 nM I found BV2 migration to be increased by approximately 20% compared to the control group, however without any statistical significance. Therefore, migratory activity of BV2 cells was significantly higher for higher apelin-13 peptide concentrations. Migration increased by 25% at a peptide concentration of 200 nM and even by 32% at 500 nM compared to the control (*figure 12*). A direct connection of apelin-13 and BV2 microglial migratory activity was shown. Higher apelin-13 concentration resulted in a higher migration of BV2 cells with an migrational increase of 32% for the maximum concentration of 500 nM apelin-13.

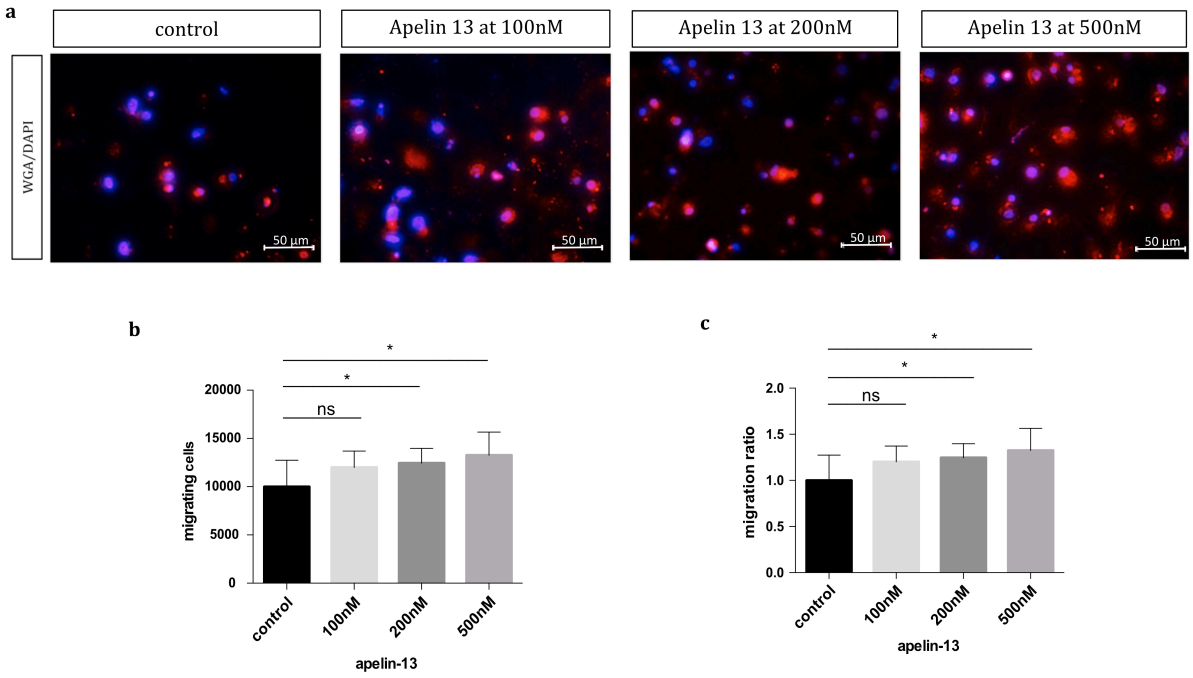

Figure 12
Apelin-13 attracts BV2 microglia in a dose dependent way. BV2 migration was tested without apelin-13 as chemoattractant = control (DMEM without FCS) and with peptide concentrations of 100, 200 or 500nM (a). Statistical significance for higher migration could be found for 200 and 500nM when compared to the control group (b), increase was about 20% for 100nM, 25% for 200nM and 32% for 500nM (c) (two-tailed student's t-test). Number of replicates n=3, sample size N=3; *, P<0,05, ns = no significance; scale bar = 50 µm.

Blue: DAPI (nuclear staining), Red: WGA (wheat germ agglutinin for membranes).

To test if macrophages behave similar to microglia, I took J774 cells instead of BV2 and performed another series of migration assays. For the tested apelin-13 concentrations of 100, 200 and 500 nM, the observed increase of J774 migratory activity was not significant when being compared to the control group. It is necessary to point out the high standard deviation in the control group of this experiment, which might explain the lack of statistical significance. Nonetheless, I analyzed J774 migration depending on different apelin-13 concentrations. An apelin-13 peptide concentration of 200 nM showed the strongest effect and was related to 43% higher migratory activity of J774 macrophages compared to the control. 100 nM of apelin-13 resulted in an increase of 31%, the highest peptide concentration, 500 nM, was related to 26% stronger migration (*figure 13*). Interestingly, the strongest increase of migratory behavior was observed at a lower apelin-13 concentration compared to BV2 cells. At 200 nM peptide concentration J774 cell migration was 43% stronger than in the control group. This might indicate that J774 macrophages are more sensitive for lower concentrations of apelin-13 peptide than BV2 microglia cells. But overall, the lack of statistical significance leads to the interpretation that there is no major effect of apelin-13 peptide on J774 migration.

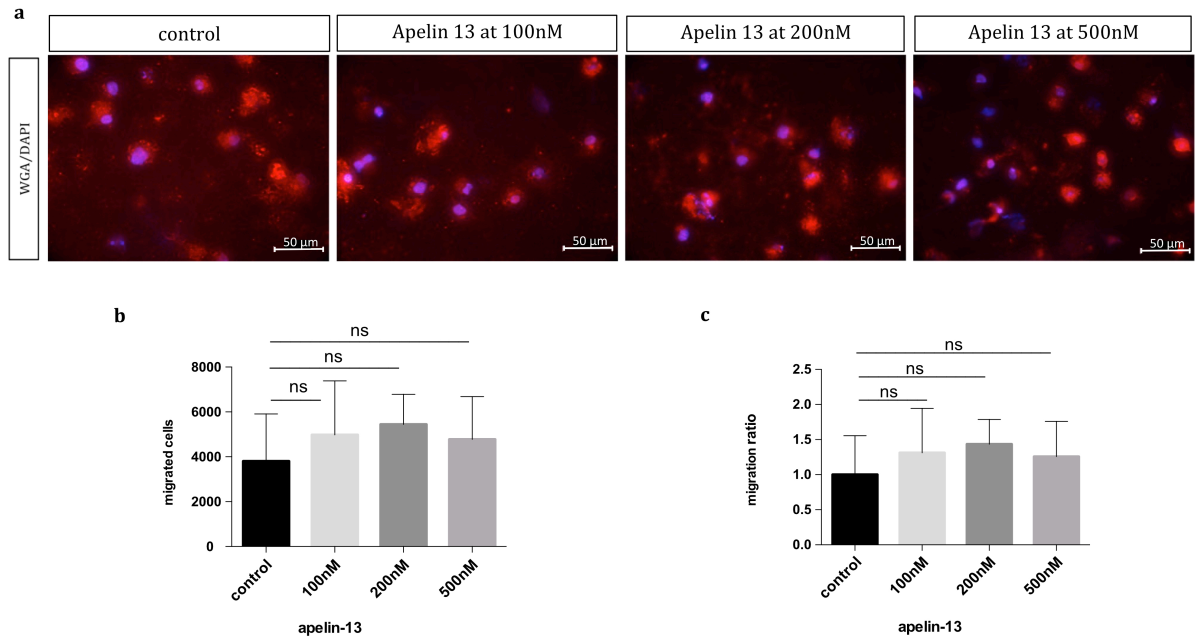

Figure 13
Apelin-13 has no significant effect on J774 macrophages. J774 migration was tested without apelin-13 as chemoattractant = control (DMEM without FCS) and with peptide concentrations of 100, 200 or 500nM (a). No significances could be found (b), increase was about 31% for 100nM, 43% for 200nM and 26% for 500nM (c), (two-tailed student's t-test). Number of replicates n=3, sample size N=3, ns = no significance; scale bar = 50 µm.

Blue: DAPI (nuclear staining), Red: WGA (wheat germ agglutinin for membranes).

The data generated in this experiment was used to detect differences in the general migratory character of both cell lines. The total amount of migrated cells either in the control and the apelin-13 group was significantly higher for BV2 than for J774 cells. Exposed to apelin-13 at 200 nM, a total number of 12.400 (control group 10.000) BV2 cells but only 5.400 (control group 3.800) J774 cells migrated (*figure 14*). Summarizing, BV2 cells seem to migrate stronger than J774 cells in general.

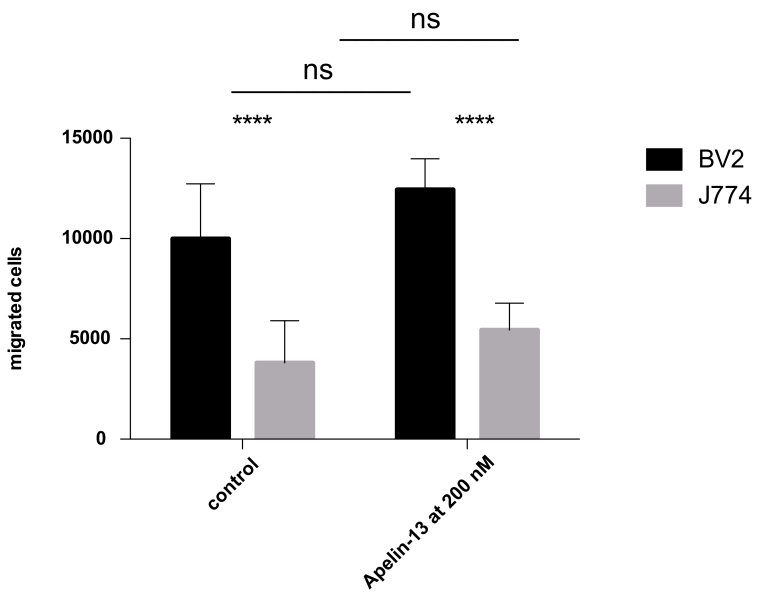

Figure 14
BV2 microglia is more migrative than J774 macrophages. The total amount of migrated cells is significantly lower for J774 macrophages compared to BV2 microglia as well in the control as in the experimental apelin-13 (200 nM) group (a). Relative change of migration at 200 nM apelin-13 was stronger for J774 macrophages (b), (Two-tailed student's t-test). ****, P<0,0001, ns = no significance.

4.3.3 Glioma cells show different effects on the migratory behavior of TAM

In the previous experiments, apelin-13 peptide was shown to attract BV2 microglia. J774 macrophages were not affected significantly by apelin-13. As already mentioned, apelin is produced in cells as a pre-proprotein consisting of 77 amino acids. Posttranscriptional processing leads to various isoforms such as apelin-13, -15, -36 or others [46]. To test if endogenous apelin peptides affect myeloid cell migration, I performed further *in vitro* experiments on migration behavior using supernatant of different human glioma cell lines (U87, GBM14 and NCH644). The supernatant contained all proteins and factors secreted by these cells. Supernatant of human glioma can be used for experiments with murine myeloid cells, as human apelin-13 was shown to be identical with mouse apelin-13 concerning the amino acid

sequence [188]. As the aim was to especially investigate the effect of apelin on myeloid cells, supernatant of APLN wildtype (APLN WT) and APLN knockdown (APLN KD) glioma cells was used and the influence on myeloid cell migration was compared. GBM14$^{APLN\ KD}$- and NCH644$^{APLN\ KD}$-cells were generated previously. *Mastrella et al.* showed 86% reduction of APLN-expression in GBM14 and 90% reduction of APLN-expression in NCH644 cells when being compared to their nonsilencing controls (NSC) [44]. U87$^{APLN\ KD}$-cells showed 90% reduction of APLN-expression [176].

In 2019, *Mastrella et al.* published data for APLN-expression in the mentioned glioma cells. The results showed U87 to be the strongest APLN-expressing cell line among the ones tested. APLN-expression of GBM14 was significantly lower than in U87 but still stronger than in NCH644 cells, that showed the lowest APLN-expression levels of the GBM cells used in my experiments (*figure 15*) [44].

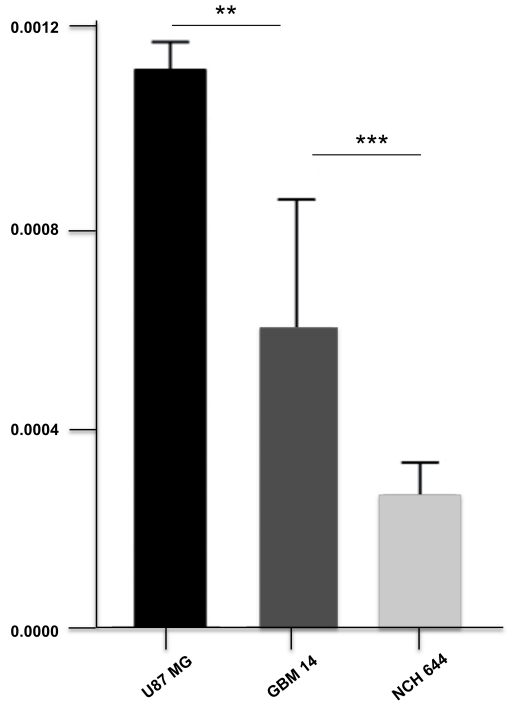

Figure 15
APLN expression levels differ significantly between glioma cell lines. U87, GBM14 and NCH644 cells all show APLN expression. U87 (number of replicates n=4) cells are related to significantly stronger expression levels than GBM14 (n=7) and NCH644 (n=4) cells. Statistical significance is indicated (one-way ANOVA). **P < 0.05, ***P < 0.005. Data adopted by *Mastrella et al,* 2019.

The glioma cell line U87 was cultured under spheroid conditions in DMEM medium, GBM14 and NCH644 were cultured as APLN WT or APLN KD cells under stem cell cultivation conditions in DMEM + F12 by my colleagues. I used the supernatant of these cells as conditioned medium for my following migration experiments on myeloid cells. For $U87^{APLN\ WT}$, which are known to have the strongest APLN expression among the cells tested (*figure 15*), a significant decrease of BV2 migration was observed in the knockdown group. Compared to APLN WT cells, the migration rate for BV2 cells was about 30% lower when being exposed to supernatant of $U87^{APLN\ KD}$ cells (*figure 16*). I assumed APLN/APLNR-signaling to be used by U87 glioma cells to attract BV2 microglia.

Mastrella et al. showed APLN-expression levels of $GBM14^{APLN\ WT}$ cells to be lower compared to $U87^{APLN\ WT}$ [44]. Concerning BV2 migration activity, I detected no difference between the APLN WT and APLN KD group (*figure 16*). Interestingly, supernatant from $NCH644^{APLN\ WT}$, which among the three tested glioma lines had the lowest APLN-expression, caused microglia migration, that was significantly stronger in the APLN KD than in the APLN WT group (50%) (*figure 16*). In this setting it seems like APLN/APLNR-signaling is even inhibiting BV2 microglia migration towards NCH644 tumor cells.

Comparing these results to the previously described experiments using pure apelin-13 peptide without glioma supernatant it was observed that migration of BV2 cells was most influenced by high apelin-13 concentrations (*figure 12*). Coherently, reduced APLN-expression in U87 glioma cells, the strongest APLN-expressing glioma cells tested, resulted in significantly lower BV2 migration.

When comparing the total number of migrated BV2 cells to the control group, it was lower for all tested GBM cells (U87, GBM14 and NCH644). This observation was found for APLN WT and APLN KD cells and led to the assumption the conditioned medium might contain some unknown migration-inhibiting factors for BV2 cells.

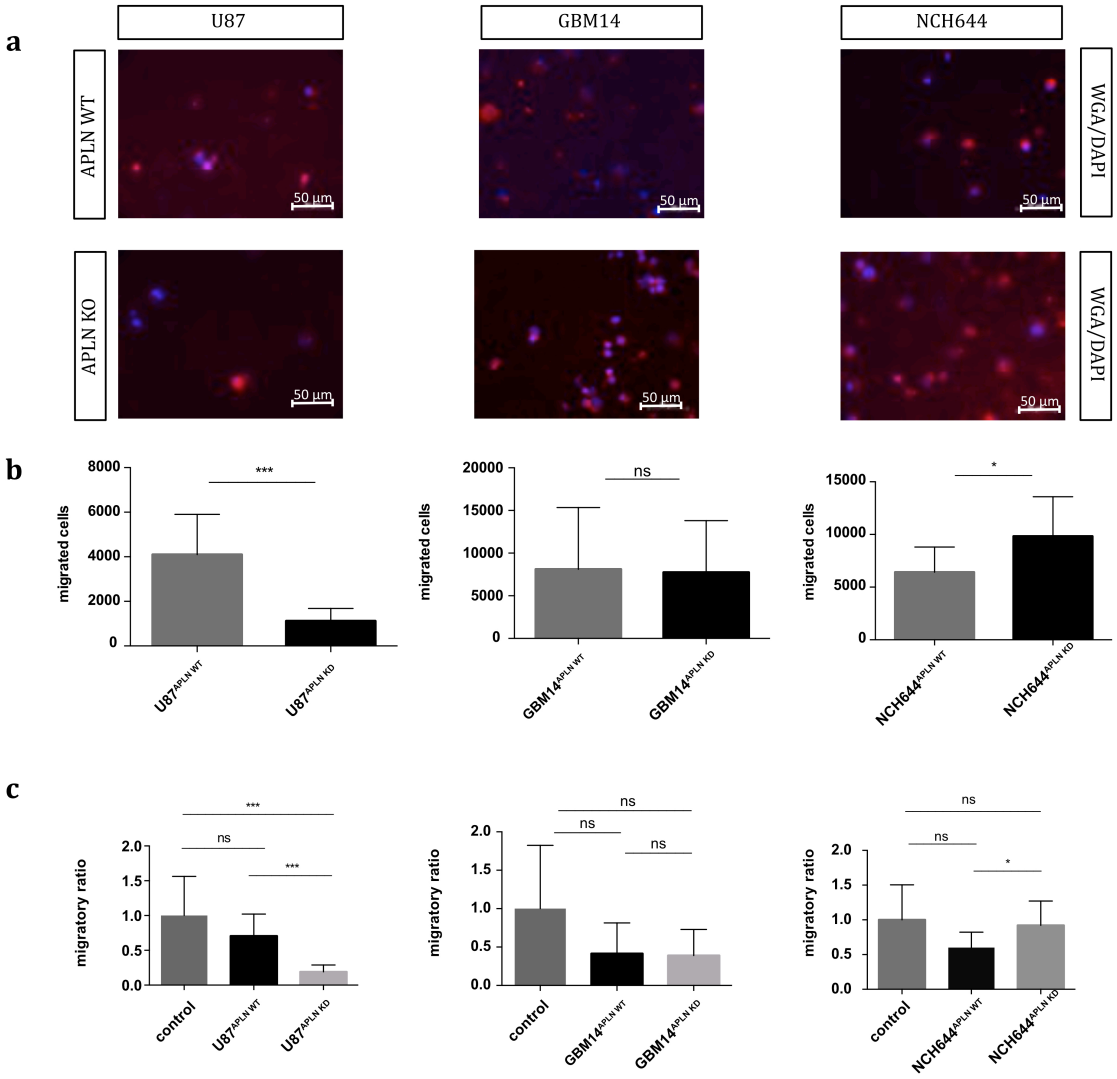

Figure 16
GBM-derived apelin has differential effects on BV2 migration. Supernatant of highly APLN-expressing U87 glioma cells (*figure* 15) shows significant reduction of migration in the APLN knockdown group. For supernatant obtained from GBM14 cells no difference between wildtype and knockdown group was detected, whereas NCH644 cell supernatant was even related to stronger migration activity in the knockdown group (a + b). Relative numbers compared to the control group are shown in (c). Number of replicates n=3, sample size N=3; Statistical significance is indicated (two-tailed Student's t-test). *P < 0.05, **P < 0.05, ***P < 0.005, ns = no significance; scale bar = 50 µm.

Blue: DAPI (nuclear staining), Red: WGA (wheat germ agglutinin for membranes).

As already described, pure apelin-13 had no significant effect on migration of J774 macrophages which might be due to the high standard deviation in the control group.

Nevertheless, I conducted the glioma supernatant experiments with this cell line as well and checked for differences between the APLN WT and APLN KD group.

For the supernatant of highly APLN-expressing U87 cells I observed no signifcant difference in J774 migration when comparing APLN WT and APLN KD supernatant (*figure 17*). Furthermore, no significant difference was found for GBM14$^{APLN\ WT}$ and GBM14$^{APLN\ KD}$ supernatant. These findings are in line with the previous results of my J774 migration experiment on recombinant apelin-13 in which I showed that the peptide has no influence on migration of J774 macrophages. Surprisingly, when testing NCH644 medium, significant differences between both groups were found. Compared to the APLN WT group, only 30% of J774 macrophages migrated in the APLN KD setup (*figure 17*).

Interpreting my results, I suggested APLN/APLNR-signaling to be irrelevant for J774 attraction in U87 and GBM14 cell gliomas. To proof relevance for APLN/APLNR-signaling on J774 migration in the context of NCH644 gliomas it would be necessary to block APLNR in J774 cells and to perform the experiment with these cells again. If APLN secreted by NCH644 cells has an impact on J774 migration, blocking APLNR in J744 would result in lower migratory activity.

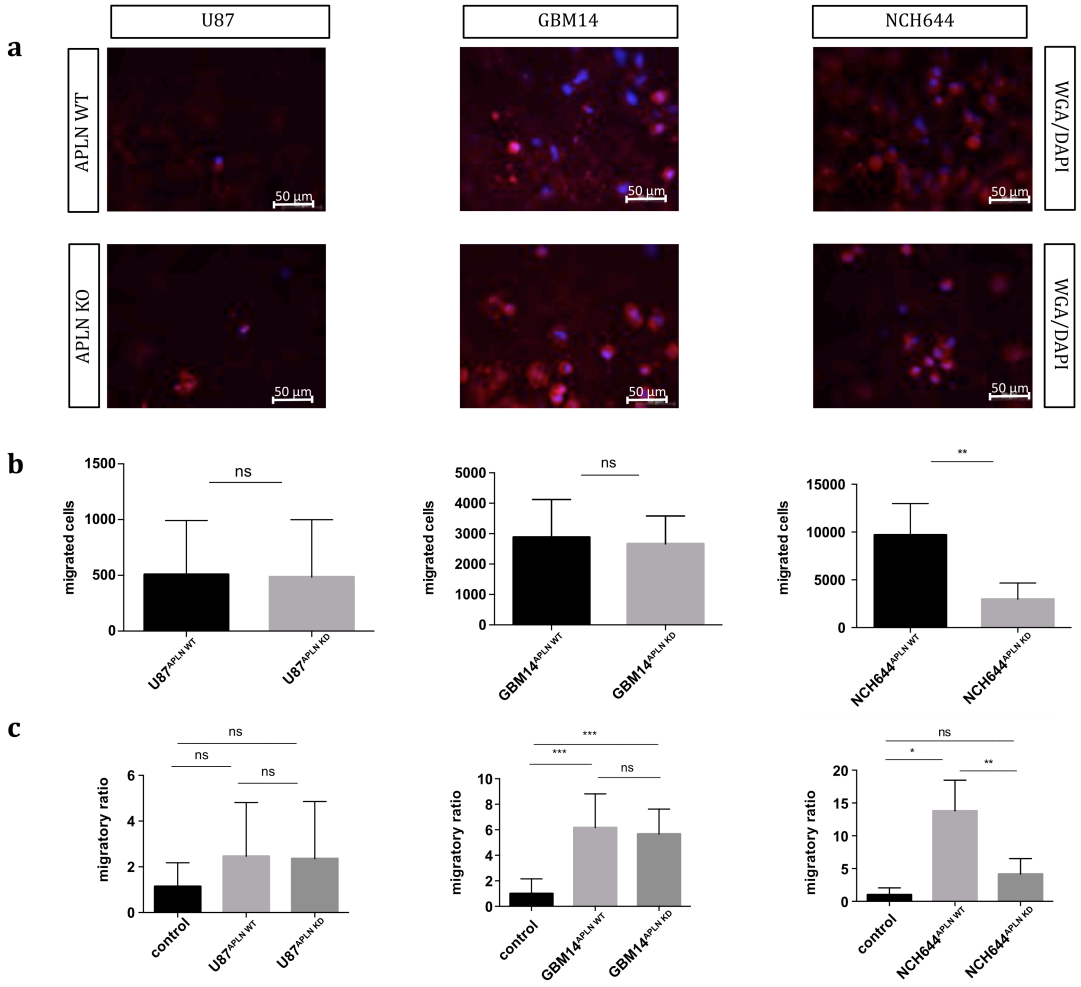

Figure 17
J774 migration is independent from APLN/APLNR-signaling in U87 and GBM14 glioma cells. Neither for highly APLN-expressing U87 glioma cells (*figure* 15) nor for GBM14 cells a difference between wildtype and knockdown group was detected. Nevertheless, NCH644 cell conditioned medium was related to significant lower migration activity in the knockdown group (a + b). Relative numbers compared to the control group are shown in (c). Number of replicates n=3, sample size N=3; Statistical significance is indicated (two-tailed Student's t-test). *P < 0.05, **P < 0.05, ***P < 0.005, ns = no significance; scale bar = 50 µm.

Blue: DAPI (nuclear staining), Red: WGA (wheat germ agglutinin for membranes).

Summarizing, my observations could speak for other isoforms of apelin and other chemoattractant factors included in the supernatant of GBM cells to influence TAM migration towards glioma, too. Therefore, whole transcriptome gene expression analysis might be performed for U87, GBM 14 and NCH 644 cells in the APLN WT

and APLN KD situation as a next step to detect the factors with a potential influence on TAM migration.

4.3.4 The culture medium does not influence myeloid cell migration

Under control conditions BV2 cells migrated stronger than J774 concerncing the total amount of migrating cells (*figure 14*). I aimed to investigate the influence of the culture medium on myeloid cell migration as glioma cells were cultured in different medium. GBM14 and NCH644 were cultured in DMEM F12 medium, whereas U87 cells were cultured in DMEM only. When focusing on the total number of migrated cells in my supernatant experiments it was noticeable that exposed to supernatant of U87 cells (cultured in DMEM only) the amount of migrating BV2 and J774 cells was lower than when being exposed to supernatant of GBM14 and NCH644 cells (cultured in DMEM F12). But comparing the control groups of DMEM and DMEM F12-cultured glioma cells I found that migratory activity of BV2 microglia as well as J774 macrophages is not significantly influenced by the culture medium (*figure 18 a+b*) and concluded that F12 includes no chemoactive substances which could be relevant for my experiments.

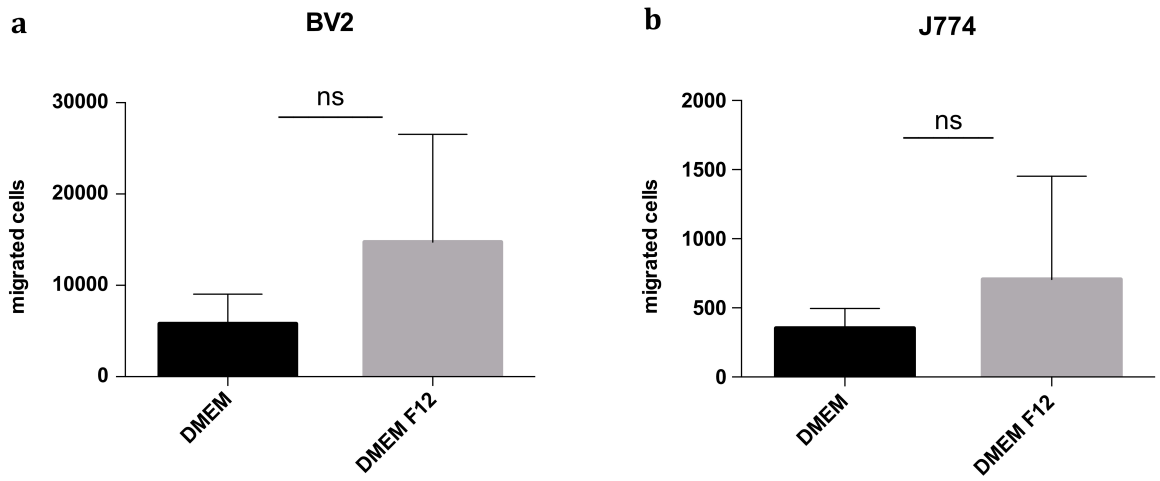

Figure 18
The culture medium (DMEM vs. DMEM F12) has no influence on migration activity of BV2 and J774 cells. Migration activity is shown for BV2 (n=3, N=2 for DMEM, n=3, N=4 for DMEM F12) (a) and J774 (n=3, N=2 for DMEM, n=3, N=1 for DMEM F12) (b), observations were not statistically significant (two-tailed student's t-test), ns = no significance.

4.3.5 The APLN antagonist apelin-F13A has no major effect on migration of BV2 microglia

For an additional experiment on the migrational behavior of microglial BV2 cells, apelin-13 antagonist apelin-F13A was used. Modification of the terminal residue of apelin-13 creates this new peptide, apelin-F13A, which acts as a competitive APLN antagonist [189]. *Mastrella et al.* could show a protective effect of apelin-F13A on glioma invasion and angiogenesis. Nevertheless, they could not show a significant decrease of TAM density in GBM-suffering mice after treatment with apelin-F13A [44]. In my *in vitro* experiments, neither I found significance for a change in microglial migratory behavior when being exposed to apelin-F13A containing medium (*figure 19*). Therefore, I underlined the findings by *Mastrella et al.*, which showed TAM density not to be influenced by administration of apelin-F13A. The antagonist does not directly influence microglial migration and therefore has no major effect on BV2 migration. But as apelin-F13A is a competitive inhibitor for apelin-13 it might affect

BV2 migration via interaction with apelin peptides secreted by glioma cells. It would be the next step to test the effect of apelin-F13A on BV2 migration when being added to U87 APLN WT and U87 APLN KD conditioned medium. This experiment would allow conclusions on whether apelin-F13A can block GBM-induced migration and should be performed as a next step.

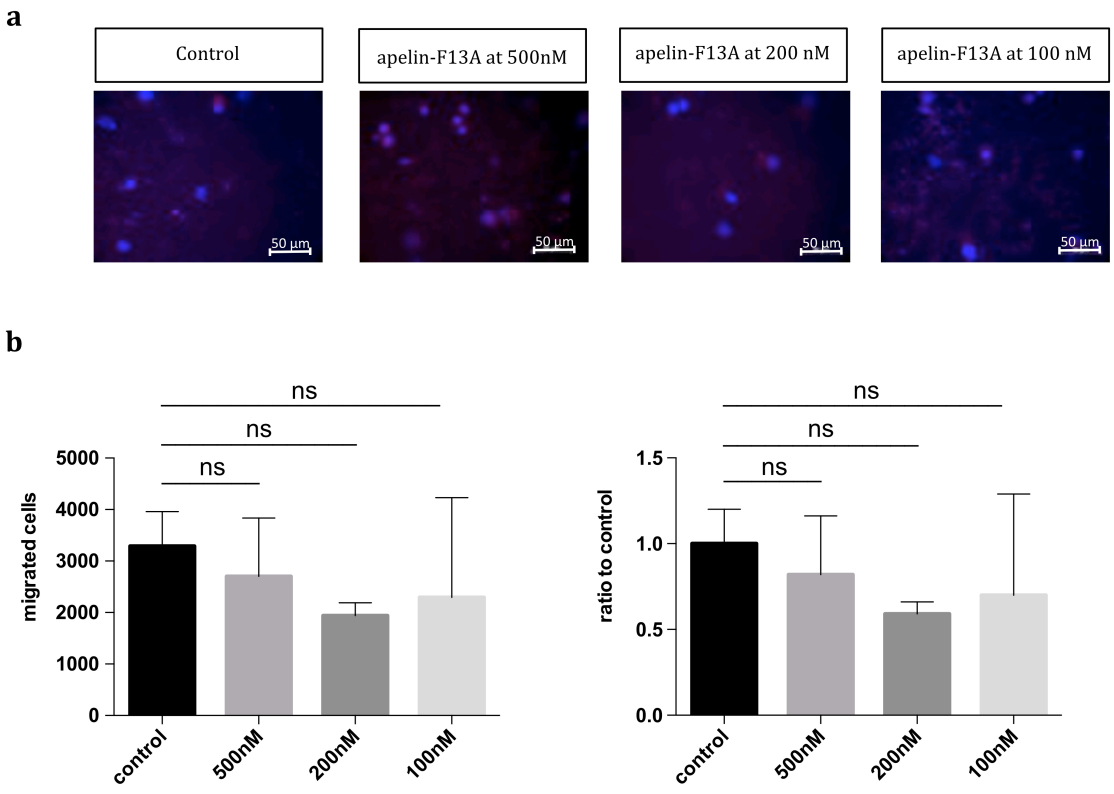

Figure 19
Apelin-F13A has no major effect on BV2 microglia migration. BV2 migration was tested without apelin-F13A as chemoattractant = control (culture medium DMEM without FCS) and with peptide concentrations of 100, 200 or 500nM (a). No statistical significances for lower migration could be found (two-tailed student's t-test) (b). Magnification 40x, n=3, N=3, ns = no significance; scale bar = 50 µm.

Blue: DAPI (nuclear staining), Red: WGA (wheat germ agglutinin for membranes).

4.3.6 Endothelial cell-derived factors do not affect migratory behavior of BV2 cells

In a last experiment a focus was put on endothelial cells. In 2017, *Harford-Wright et al.* showed APLN to be expressed in endothelial cells during the angiogenesis in GBM. *Saint-Geniez et al.* found stronger proliferation in these APLN- and APLNR-expressing cells when being exposed to apelin. In a tumoric context, this could result in stronger tumor angiogenesis when being exposed to apelin derived from endothelial cells [96] [190] [191]. Thus I wondered if the migratory behavior of myeolid cells could be influenced by apelin derived from endothelial cells. For my experiment I used human umbilical vein endothelial cells (HUVEC) at passage numbers four and seven, originally cultured in endothelial growth medium-2 (EGF-2) by *Dr. R. Kälin* and obtained from Cambrex® [187]. As *figure 20* shows, HUVEC did not influence the migrational behavior of BV2 cells significantly compared to the control group with medium only.

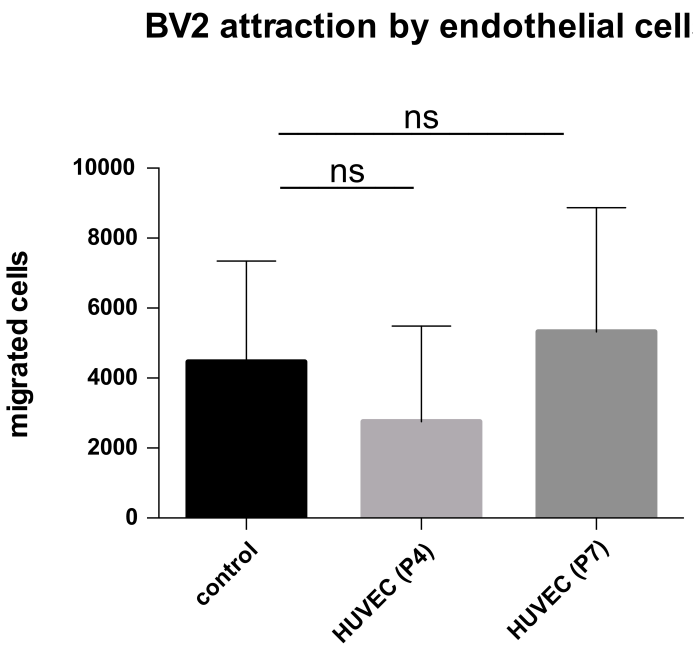

Figure 20
Endothelial cells do not affect migration behavior of BV2 cells. BV2 migration was investigated by using the supernatant of different HUVEC endothelial cell passages (P4 and P7); n=3, N=2; No significance was found (two-tailed Student's t-test), ns = no significance.

5. Discussion

Previous data obtained from a mouse model for GBM suggested that APLN plays a role in controlling TAM density *in vivo* (*figure 2*, unpublished data by *R. Kälin et al.*). Based on this finding, I investigated in my thesis if myeloid cell migration could be directly affected by the apelin-13 peptide or glioma cell-derived APLN. Towards this aim, I analyzed tissue of primary GBM mouse models and performed *in vitro* cell migration assays.

5.1 U87 glioma cells attract myeloid cells through APLN-signaling

Several factors like MCP-1, MCP-3, SDF-1 or CSF-1 have been identified to attract myeloid cells towards the tumor [153 162 163 164 166]. APLN has not been mentioned among these chemoattractants so far. The research group of *Dr. Roland Kälin* used U87 glioma cell xenografts to investigate the effect of APLN on myeloid cells. Combining selective knockdown of APLN-expression in the tumor cells and knockout of APLN in mice he distinguished how glioma- and mouse-derived APLN influences myeloid cell density. *Figure 2* shows the unpublished data. The lowest density of TAM was observed in $APLN^{KO}$ mice infiltrated with apelin-deficient $U87^{APLN\ KD}$ glioma cells. Nevertheless, $APLN^{WT}$ mice suffering from $U87^{APLN\ KD}$ glioma showed an amount of TAM that was similar to the density of TAM in the aforementioned setting, an observation that led to the assumption of tumor-derived APLN to be responsible for TAM attraction towards gliomas. This hypothesis was supported by data from $APLN^{KO}$ mice injected with $U87^{APLN\ WT}$ glioma cells, as this group showed a significantly higher density of TAM compared to the $U87^{APLN\ KD}$ group.

The obtained results suggested a role for APLN peptides in the context of TAM attraction towards glioma. It prompted us to further investigate the underlying cell biological mechanisms.

5.2 Strong APLN expression levels of glioma cells are directly correlated with high density of TAM in the tumor microenvironment in vivo

As a high amount of glioma-derived apelin was correlated with high TAM density in xenografts of the GBM cell line U87, we aimed to investigate the relevance of apelin in controlling TAM density for other glioma subtypes, too. Therefore, new primary GBM mouse models generated by our research group were used (*Mastrella et al. 2019* [44]*, Volmar et al. 2021* [192]). They represent different GBM subtypes or contain typical drivers for human GBM (cdkn2aEGFRvIII) [44]. Using these novel GBM models, I found that the level of APLN expression *in vitro* directly correlates with TAM density *in vivo* (*figures 5-8*). *Mastrella et al.* found the apelin-receptor (APLNR) to be expressed in the tumor center as well as in the invading zone. Interestingly, APLN itself was expressed in the tumor core but could not be detected at the tumor border [44]. As apelin is a secreted peptide, I tested how far an apelin-specific effect on myeloid cells could reach. I defined four different areas of the glioma-infiltrated mouse brain to measure TAM density: the tumor center, the invading zone, the tumor cell free juxtatumoral border (peritumoral region) and the contralateral brain region as a control (*figure 4*). Some regions could not be defined in some GBM models as the tumor mass was too compact (for example in NCH644) lacking an invading zone or too invasive (GBM13), so no peritumoral region could be defined. Within the tested GBM samples, cdkn2a^{KO}EGFRvIII glioma cells showed the highest APLN expression, whereas GBM13 glioma cells showed the lowest expression levels. I found that the density of TAM directly correlates with the APLN expression levels in all regions, a finding that supported the assumption of a direct role for APLN in TAM migration and density. It is also notable, that the direct correlation of APLN expression levels and TAM density was shown for the contralateral hemisphere, too. So far, APLN/APLNR-signaling was assumed to be relevant especially for tumor angiogenesis in the microenvironment of gliomas. These new findings also suggested an effect of APLN on myeloid cell density including not only the tumor microenvironment, but also the whole glioma-exposed brain. This is consistent with another striking observation. Only cdkn2a^{KO}EGFRvIII mouse models showed a significant difference in the area covered by Iba1 between all regions. For GBM14, GBM 13 and NCH644 mouse models I could not show significantly different myeloid

cell density between the four brain regions (*figure 9*). In this context it has to be mentioned that Iba1 is a marker for myeloid cells in general, not only for TAM [193]. Thus, the measured amount of myeloid cells includes physiological microglia and macrophages as well as TAM.

In summary, my data supports the following interpretation: glioma-derived apelin potentially has some influence on a subset of myeloid cells, increases their density in the microenvironment and thereby might promote the invasiveness of the tumor. Additionally, glioma-derived apelin might influence myeloid cell density in the contralateral side of the brain as well. It is also relevant to emphasize that so far my experiments have not distinguished between microglia and macrophages in the tumor environment. Using new markers like CD45, CD49d or Tmem19 could help to investigate the composition of TAM in the microenvironment of GBM more detailed [129 130 131 193]. This has relevance as I found hints for a different behavior and function of microglia and macrophages in the context of GBM in my migration experiments that will be discussed later in my thesis.

5.3 BV2 and J774 cells express APLNR

To show that BV2 and J774 cells are able to respond to APLN signals, expression of the receptor APLNR had to be proved. I selected two available myeloid cell lines and analyzed their apelin receptor expression. Therefore quantitative PCR against APLNR was performed. The results showed APLNR to be expressed in BV2 and J744 myeloid cells. Higher levels were detected in BV2 microglia cells (*figure 10*).

As I found that APLNR-expression levels were lower in BV2 and J774 cells compared to cdkn2a^{KO}EGFRvIII glioma cells, I wondered if the APLNR expression levels are stable or could be modulated by stimulation with LPS, which is causing inflammation and is known to be a strong activator of myeloid cells [183]. Stimulation with LPS did not result in stronger APLNR and APLN expression in BV2 and J774

cells. Interestingly, the receptor could not be detected in microglia after LPS treatment anymore (*figure 10*). Myeloid cells fulfill an immunological function by protecting the body from harmful influences and diseases [194]. Immune stimulation by administration of LPS triggers this function. In 2009, *Leeper et al.* showed reduced inflammation activity of macrophages after apelin-13 treatment [184]. In 2014, *Obara et al.* reported that apelin-13 inhibits LPS-induced production of pro-inflammatory cytokines in J774.1 macrophages [185]. Both studies suggest apelin to inhibit inflammatory reactions. In my work I assume that LPS reduces APLNR expression in myeloid cells and therefore their sensitivity for apelin. As we suggested that APLN/APLNR-signaling is of certain relevance in a GBM tumor setting, in which myeloid cells lose their classical immunological functions and even support the malignancy of the tumor [91] [92], my findings support the hypothesis that the APLN/APLNR pathway is downregulated when microglia and macrophages are put in an inflammatory setting by treatment with LPS.

Summarizing, LPS might reduce the anti-inflammatory effect of apelin on myeloid cells by down-regulation oft APLNR.

5.4 Apelin-13 peptide attracts microglia

Fetal calf serum (FCS) is needed in cell culture for optimal cell growth and includes several chemotactic factors [195]. A multitude of these factors can have an effect on cell migration. Therefore, its influence on myeloid cell migration behavior was investigated first to evaluate the optimal conditions for experiments with APLN peptide and APLN-containing glioma cell medium. As *figure 11* shows, I could not find a significant influence of FCS on BV2 migration. Nevertheless, the sample size was small (n=3) and a tendency to higher migration in FCS-containing medium was observed, so I decided to test myeloid cell migration without FCS in the attractive medium. Both myeloid cell lines, BV2 and J774 cells, showed sufficient growth and vitality under FCS-free conditions for the time of 24 hours. This time was needed to

perform the migration experiments. The optimal cell number of 100.000 cells was established following a protocol for invasion assays by *Justus et al.* from 2014 [186].

So far, myeloid cells like microglia or macrophages were shown to be part of the microenvironment of gliomas [155]. Nevertheless, there is still limited knowledge about how each subtype of myeloid cells contributes to the composition of TAM and whether they fulfill different functions. Similarities and differences in their behavior in context of gliomas have just started to be investigated [152] [154] [155]. To address this point, microglia and macrophages were used for migration assays. As a first step, the influence of apelin-13 peptide on each myeloid cell line was investigated. Using BV2 microglia, apelin-13 in a concentration of 200 and 500 nM caused significantly higher microglial cell migration (*figure 12*). I could show that this isoform of the apelin peptide influences BV2 migration. Transferring these findings in the setting of a patient suffering from GBM one can resume, that at least one tumor-derived apelin isoform might contribute to microglial attraction towards the tumor by using the APLN/APLNR-pathway.

Next step was to investigate whether migration of J774 macrophages might be influenced by apelin-13 as well. As *figure 13* shows, migration of macrophages seemed increased when being exposed to apelin-13 concentrated at 100, 200 and 500 nM, but anyhow without statistical significance. As no major effect of apelin-13 on J774 migration was detected, I concluded that attraction of J774 cells towards GBM might depend on other isoforms of apelin or even on other chemoattractants.

The total amount of migrated cells differed between BV2 and J774. Even without apelin-13, microglia seemed to be more migrative than the investigated macrophages (*figure 14*). The significant increase of BV2 migration under apelin-13 exposure compared to the non-significant increase of J774 might be a result of stronger APLNR expression in this cell line. One could assume a stronger effect of apelin-13 on cells with a high amount of APLN receptor. Consequently, it leads to the assumption that microglia and not macrophages might be the dominant myeloid cell group in the context of TAM migration towards gliomas.

5.5 GBM-cell conditioned medium triggers migratory effects on microglia in an APLN-dependent manner

The previous results were obtained after stimulation with recombinant APLN and suggested APLN to act as a direct stimulus for myeloid cell migration. In order to obtain data from a more complex *in vitro* setting, supernatant of three different glioma cell lines in culture – U87, GBM14 and NCH644 – was used. For each cell line, the migration of BV2 and J774 was compared when being exposed to conditioned medium from either APLN-expressing GBM cells or APLN-deficient GBM cells. The composition of this conditioned medium was not analyzed and can contain plenty of substances that remain unknown. The experiment allows conclusions about the influence of APLN on BV2 and J774 migration under experimental conditions, that more closely recapitulate the pathological situation.

As shown in *figure 3,* GBM cells differ in the amount of APLN expression. In 2019, *Mastrella et al.* showed GBM14 to express the protein stronger than NCH644. U87 glioma cells are correlated with the highest APLN expression levels of all three tested GBM cell lines (*figure 15*) [44]. As a result of my previous findings, U87 is assumed to have the strongest influence on myeloid cell migration followed by GBM14 and NCH644. The aim of the following migration assay experiments was to determine, whether loss of APLN expression in glioma cells – by selectively knocking down the APLN gene – results in different migratory behavior of myeloid cells.

In a first set of experiments, migration of BV2 microglia cells was investigated when being exposed to U87, GBM14 and NCH644 glioma supernatant. Supernatant of APLN wildtype or APLN deficient GBM cells was tested and results were compared. For U87, the strongest APLN producing glioma cell line tested, a significant decrease of BV2 migration activity could be detected in the knockdown group (*figure 16*). This finding supports the hypothesis that APLN is a factor of microglia chemoattraction towards the tumor. GBM14 supernatant did not show any difference between both groups. Moreover, experiments with NCH644 supernatant, which according to my qPCR results was assumed to contain the lowest APLN levels, showed the reverse

effect. An increased migration activity for microglia in the APLN knockdown group was found.

Whereas the results for U87 perfectly underline the assumption of APLN being a chemoattractant for microglia, interpretation for GBM14 and especially NCH644 supernatant is more complicated. It is established that various chemoattractant factors contribute to the complex process of myeloid cell attraction towards glioblastomas. In 2015, *Hambardzumyan et al.* gave a summary of all relevant recruitment factors for tumor-associated macrophages (TAMs) in gliomas. MCP-1, SDF-1 and many others are known so far [153]. In this context it seems obvious that recruitment of TAMs does not only depend on one factor. It is a complex process including many molecules and pathways. I suspect APLN to have a special relevance for microglial migration in context of tumor subtypes with high APLN expression like U87. For that reason, loss of APLN results in lower microglia recruitment (*figure 16*). In consequence, it seems that microglia needs high levels of the peptide for migration. GBM14 gliomas could use other pathways more than APLN/APLNR-signaling to attract myeloid cells so that these cells can increase tumor growth and invasiveness [154] [152]. The stronger microglia migration in the NCH644 APLN knockdown group contradicts my working hypothesis. Interaction of apelin with other components of the NCH644 conditioned medium could be responsible for this observation and was not investigated in my study. Furthermore, a very low APLN level could also act as an inhibitor of BV2 attraction, too. In this assumption, APLN could have two effects on microglia: attracting microglia at high APLN levels (U87) and inhibition of microglia recruitment at low levels (NCH644). This inhibiting effect would be reduced by APLN knockdown, which finally results in stronger microglia recruitment in the APLN knockdown situation for low APLN expressing NCH644 glioma cells. In this model, TAM recruitment in GBM14 and NCH644 gliomas depends much more on other pathways than on APLN/APLNR-signaling. I conclude that apelin is just one factor that can attract myeloid cells. Other chemoattractive factors might be more important, at least in some GBM.

It is also worth mentioning that different GBM cells might express different isoforms of APLN. These isoforms might affect microglia differently. This could be an

explanation for the observed differences in BV2 migration when being exposed to conditioned medium of U87, GBM14 or NCH644 glioma cells. To further investigate different influences of apelin isoforms on microglial migration it would be a next step to perform mass spectrometry to identify the apelin isoforms expressed by different GBM cells.

5.6 Migration of macrophages is independent from APLN/APLNR-signaling

The experiments outlined above (using BV2 cells) were repeated with J774 macrophages, but no significant influence of apelin-13 on these cells was found (*figure 13*). Nevertheless, more information about the behavior of J774 in context of gliomas was needed so I used glioma cell supernatant on J774, too. As described before, the effect of APLN-deficient U87 glioma cells on BV2 microglia was significant. For J774 macrophages, I could not show any significant change of migration when being exposed to APLN-deficient U87 supernatant. The migration rate observed was similar in the group of macrophages exposed to conditioned medium received from APLN-expressing U87 cells compared to medium of APLN-knockdown glioma cells. Also, there was no significant migratory effect found for J774 macrophages when being exposed to APLN-deficient or -producing conditioned medium of GBM14 glioma cells (*figure 17*). However, reduction of macrophage migration was detected for NCH644 APLN-deficient conditioned medium (*figure 17*). It seems that at least for this glioma subtype, APLN/APLNR-signaling might contribute to the attraction of macrophages towards the tumor. It is essential to repeat the experiment after blocking APLNR in J774 cells. If APLN/APLNR-signaling is relevant for macrophage attraction towards NCH644 gliomas, reduction of migration should be lost upon blockage of APLNR in J774 cells.

The higher APLNR expression levels of BV2 cells compared to J774 cells (*figure 10 a*) support the following conclusion: APLN/APLNR-signaling has relevance for BV2 microglia as these cells are related to higher APLNR expression compared to J774 macrophages. This was shown for recombinant apelin-13 as well as for supernatant

of strong APLN expressing U87 glioma cells. J774 macrophages show very low expression levels of APLNR (*figure 10 a*) and do not respond to recombinant apelin-13. Only supernatant of low APLN expressing NCH644 cells seemed to have an impact on J774 migration, although this observation needs further investigation.

5.7 Microglia reduce migratory activity in GBM environment whereas macrophages increase migration

In my migration experiments, myeloid cells were either exposed to conditioned medium of APLN-expressing or APLN-deficient GBM cells. Pure DMEM medium served as a control for experiments with U87 cells, whereas DMEM F12 was used as control medium for GBM14 and NCH644 glioma cells. Notably, compared to these control groups, BV2 migration was consequently lower under exposure to glioma-conditioned medium (*figure 16 c*). Migration experiments using J774 macrophages provided different results. These cells, compared to the control group, reacted with stronger migration activity when being exposed to glioma cell-conditioned medium (*figure 17 c*).

It seems that macrophages increase their migratory activity in the presence of gliomas, whereas microglia show reduced migration. Microglia might tend to keep their immunological and protective function more than macrophages. This is in line with previous findings by *Annovazzi et al.* who described the number of microglia cells to be independent from the glioma malignancy grade whereas macrophages are predominant and higher concentrated in high-grade gliomas [196] [197]. Although microglia also supports tumor progression, they seem to keep a phagocytosing function, too [196]. In addition, *Brandenburg et al.* demonstrated macrophages to be primarily activated by gliomas whereas microglia are almost unaffected [198]. Based on my findings, I assumed that macrophages are mobilized easier in a GBM environment than microglia. According to my previous experiments, APLN/APLNR-signaling is not part of this phenomenon in J774 cells. Other chemoattractants included in glioma cell supernatant might therefore be responsible for increased

migration of macrophages. BV2 microglia, although related to lower migration in the environment of GBM in general, depend on APLN/APLNR-signaling as one of several pathways used by gliomas to attract microglia. This assumption furthermore implicates that the majority of TAM are macrophages and is in line with findings by *Koshkaki et al.* who described tumor-associated macrophages to be the most common cell type in the peritumoral area and in the tumor center [199]. It is a task in further studies to investigate the composition of TAM in the glioma environment in more detail. Recently, new markers such as microglia-specific transmembrane protein 119 (Tmem119) and CD49d have been found and described to distinguish microglia from macrophages [130] [131]. This allows further *in vivo* experiments to investigate the microenvironment of gliomas and to draw conclusions on the input of different TAM on GBM.

5.8 Culture medium has no influence on migratory activity of myeloid cells

U87 cells were cultured in DMEM whereas GBM14 and NCH644 cells were cultured in DMEM F12 medium, both enriched with fetal bovine serum (FBS), non-essential amino acids and penicillin-streptomycin antibiotics. In the DMEM F12 medium, epidermal growth factor (EGF) and fibroblastic growth factor were added. Interestingly, the absolute number of migrated myeloid cells seemed lower for DMEM-only cultured U87 cells than for glioma lines cultured in F12 medium (*figure 16* and *figure 17)*. F12 medium consists of DMEM with some additional supplements. One could assume that these supplements promote cell mobility, which might result in stronger migration in F12 medium than in DMEM. So I compared BV2 and J774 migration under control conditions when being exposed to either DMEM or DMEM F12 medium but did not find any statistical significance for differences and therefore for a relevant impact of the culture medium on myeloid cell migration (*figure 18*).

5.9 Neither the APLN antagonist apelin-F13A nor endothelial cell-derived factors influence BV2 migration

The competitive apelin-13 antagonist apelin-F13A was shown to reduce angiogenesis and tumor invasion of gliomas [44]. *Figure 19* does not show a significant effect of apelin-F13A on BV2 microglia, an observation that is in line with unpublished data by *G. Mastrella,* which describes that infusion of apelin-F13A does not reduce the intratumoral accumulation of myeloid cells in murine GBM models. Apelin-F13A should be added to GBM conditioned medium in a next experiment. As competitive inhibitor, F13A might affect myeloid cell migration via interaction with GBM-secreted peptides.

Finally, endothelial cells and their influence on microglia migration were investigated, but no hint for an attractive effect on these myeloid cells could be found (*figure 20*). *Mastrella et al.* showed APLN/APLNR-signaling to influence tumor angiogenesis and glioblastoma cell invasion [44]. As described previously, microglia seems to be attracted to the GBM microenvironment by APLN/APLNR-signaling, at least in some GBM entities. However, no evidence for microglia to be attracted to endothelial cells via this pathway and to contribute to tumor angiogenesis could be found.

Summarizing my study, I found that high APLN expression levels of gliomas are directly correlated with high density of TAM in the microenvironment of the tumor. I furthermore demonstrated that APLN/APLNR-signaling is part of the attraction of microglia towards gliomas, at least in some GBM subtypes. Nevertheless, several other pathways are described and might be more relevant. Finally, I observed GBM-exposed macrophages to be more migrative than microglia. Consistent with recently published studies, I conclude that macrophages are the predominant myeloid cell type in the microenvironment of gliomas and are therefore of special interest as a target for future therapies against gliomas.

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Affidavit

Eidesstattliche Versicherung

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

Glioblastoma cell-derived apelin has differential effects on myeloid cell migration

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Ort, Datum Jonathan Enders, geb. Muffler