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Vorstand: Prof. Dr. med. Bernhard Zwißler

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The IL-1 Receptor Antagonist Anakinra Ameliorates Glioblastoma Aggressiveness by Abrogating the Inflammatory Crosstalk between Tumor and Immune Cells

vorgelegt von:

Tingting Wu

aus:

Hunan, China

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Mit Genehmigung der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

First supervisor:	Prof. Dr. Dr. med. Simone Kreth
Second supervisor:	Prof. Dr. rer. nat. Jürgen Bernhagen
Third supervisor:	Priv. Doz. Dr. Jan-Michael Abicht

Dean: **Prof. Dr. med. Thomas Gudermann**

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Summary

Glioblastoma multiforme (GBM) is the most devastating tumor of the brain that accounts for 48.3% of primary malignant brain tumors. The current standard care for GBM patients is multimodal therapy combining surgical resection, radiotherapy and chemotherapy. Over the years, extensive research was conducted to explore novel treatment strategies for GBM. However, little effect has been achieved and the prognosis remains dismal, with a median survival of 12-15 months and a five-year survival rate of 6.8%. Inflammation is characterized as a major driver of all stages of cancer development. The intricate network of GBM and infiltrating immune cells boosts inflammatory cytokine production, resulting in an inflammatory tumor microenvironment that accelerates GBM progression. IL-1β has emerged as a critical pro-tumorigenic cytokine that amplifies the inflammatory cascade in the GBM microenvironment. Therefore, targeting IL-1β signaling might attenuate the tumor-promoting inflammation and reduce GBM malignancy. Anakinra is a recombinant human IL-1 receptor antagonist (IL-1Ra) that has been approved for the treatment of autoimmune diseases with a good safety profile. We hypothesized anakinra might be a promising novel therapeutic candidate for GBM patients.

In this study, we conducted in vitro experiments using a GBM cell line, GBM primary cells and human peripheral blood mononuclear cells (PBMCs) to elucidate the inflammatory network between GBM and immune cells. Firstly, the pro-inflammatory and tumor-promoting role of IL-1β in the GBM microenvironment was validated. IL-1β stimulation significantly upregulated the pro-inflammatory cytokine expressions, IL-1β, COX2, CCL2, and IL-8, in both GBM cells and PBMCs. Functional assays, including proliferation assay, wound healing assay, invasion assay and chemotaxis, have revealed that IL-1β induced a more aggressive GBM phenotype with increased proliferation and migration. To further investigate the crosstalk between GBM and immune cells in the tumor microenvironment, we established a co-culture model

consisting of GBM cells and PBMCs. Similar to IL-1β treatment, the GBM-PBMC interplay without any additional stimuli was able to induce increased expression levels of pro-inflammatory cytokines and of the transcription factor STAT3 in GBM cells Moreover, enhanced tumor proliferation, migration and reduced apoptosis was observed. Application of anakinra attenuated pro-inflammatory cytokine expression, mitigated proliferation and migration, and induced apoptosis in GBM cells in both experimental settings, IL-1β stimulation and co-culture. Moreover, anakinra dampened pro-inflammatory signaling in co-cultured T cells without affecting cytotoxic effector molecules.

Taken together, anakinra proved as an effective anti-tumor approach in vitro that ameliorated GBM aggressiveness by abrogating inflammatory signaling in the crosstalk of GBM and immune cells. These experiments may pave the road for new approaches in the treatment of GBM.

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List of abbreviations

APC	Antigen-presenting cell
BBB	Blood brain barrier
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CCL2	C-C motif chemokine ligand 2
CNS	Central nervous system
COX2	Cyclooxygenase-2
CSF	Cerebrospinal fluid
CT	Computed tomography
CTL	Cytotoxic T lymphocyte
DMEM	Dulbecco's modified Eagle's medium
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-Linked Immunosorbent Assay
FCS	Fetal calf serum
FMI	Forward Migration Index
G-CIMP	Glioma-CpG island methylator phenotype
GBM	Glioblastoma multiforme
HBSS	Hanks' Balanced Salt Solution
HIF1	Hypoxia-inducible factor 1
IDH	Isocitrate dehydrogenase
IFNγ	Interferon γ
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-17	Interleukin-17
IL-1R	IL-1 receptor
IL-1Ra	IL-1 receptor antagonist
IL-22	Interleukin-22
IL-6	Interleukin-6
IL-8	Interleukin-8
MCP-1	Monocyte chemoattractant protein-1
MGMT	O^6-alkylguanine DNA alkyltransferase
MHC	Histocompatibility complex
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
NEAA	Non-essential amino acids
NF-κB	Nuclear factor kappa B
NOMID	Neonatal-onset multisystem inflammatory disease
OS	Overall survival
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PFS	Progression free survival

PGD_2	Prostaglandin D2
PGE_2	Prostaglandin E2
$PGF_{2\alpha}$	Prostaglandin F2α
PGI2	Prostacyclin
qRT-PCR	Quantitative real-time polymerase chain reaction
RA	Rheumatoid arthritis
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
STAT3	Signal transducer and activator of transcription-3
TAM	Tumor-associated macrophage
TBST	Tris-buffered saline with Tween-20
Th	Helper CD4+ T cell
TME	Tumor microenvironment
TMZ	Temozolomide
Treg	Regulatory T cell
TXA2	Thromboxane A2
VEGF	Vascular endothelial growth factor

1. Introduction

Glioblastoma multiforme (GBM) is the most commonly occurring and aggressive malignant primary tumor of the central nervous system (CNS). Despite the advances in multimodal therapy, the prognosis of GBM still remains dismal. Inflammation is a well-characterized driver of tumor progression[1, 2]. Anakinra is a recombinant interleukin-1 receptor antagonist (IL-1Ra) that blocks the IL-1-mediated inflammatory signaling and has been approved for the treatment of autoinflammatory disorders. This thesis addresses anakinra as a new therapeutic candidate for GBM.

1.1 Glioblastoma multiforme

1.1.1 Epidemiology

According to the World Health Organization (WHO) classification[3], glioblastoma is regarded as a grade IV glioma that accounts for 48.3% of primary malignant brain tumors and 57.3% of all gliomas[4]. Even the less aggressive grade II or III gliomas could progress to the most malignant glioblastoma within 5-10 years[5]. International studies have reported an approximate GBM incidence rate of 0.59-5/100,000 population every year[6]. However, recent studies have revealed an increasing trend of incidence in many countries[7, 8]. GBM occurs more commonly in the elderly than younger adults, with a median diagnostic age of 64. The average annual incidence rate of young people at the age of 20-34 was 0.46/100,000 population. However, the number increased up to 15.29/100,000 population at 75-85 years of age[4]. Gender also accounts for an important factor. Men are more susceptible to have GBM than women by 1.58 times[4].

1.1.2 Molecular markers

Genomic analyses have identified various genetic, epigenetic or transcriptional

alterations in GBM. A homogeneous morphologic category of GBM may exhibit significant molecular heterogeneity, which appears to influence the different responses to anti-tumor therapy as well as the ultimate prognosis. A number of molecular markers have been identified in GBM. The most important ones are O^6-alkylguanine DNA alkyltransferase (MGMT) methylation, Isocitrate dehydrogenase(IDH) mutation and Epidermal growth factor receptor (EGFR) alterations.

MGMT is a protein that repairs DNA by removing the alkyl groups from O6 guanine, therefore protecting against potential pro-carcinogenic DNA damage, however, also counteracting the effect of alkylating chemotherapy drugs, such as temozolomide (TMZ)[9]. As TMZ is a part of standard care for GBM patients, TMZ resistance induced by MGMT largely contributes to therapy failure. The epigenetic silencing of methylated MGMT leads to MGMT expression deficiency and compromises the DNA repair mechanism, which occurs in approximately 50% of GBM patients[10, 11]. MGMT methylation is known as a prognostic and predictive factor in GBM treatment and has a significant association with enhanced overall survival (OS) and progression free survival (PFS) in GBM patients[12, 13]. Additionally, it is also reported that MGMT methylated tumors exhibited favorable outcomes in response to TMZ as well as concurrent treatment of TMZ and radiotherapy, but not in the radiotherapy alone[14, 15].

IDH mutation is one of the most frequent and critical genetic alterations in gliomas, especially in low-grade gliomas and secondary GBMs. IDH enzymes have three isoforms, of which IDH1 localizes in the cytoplasm, whereas IDH2 and IDH3 function in the mitochondria. IDH mutations are predominantly somatic and universally heterozygous, catalyzing α-ketoglutarate to aberrant production of 2-hydroxyglutarate[16]. The IDH-wildtype glioblastoma accounts for about 90% of all GBM cases and corresponds mostly to primary glioblastoma in elderly patients. The IDH-mutant glioblastoma takes up only 10% of all GBM and relates closely to

secondary glioblastoma and younger patients[3]. Numerous studies have reported that IDH-mutant patients tend to have more favorable outcomes in comparison with the IDH-wildtype regarding both low-grade and high-grade gliomas [17-19].

EGFR alterations have been found in association with multiple cancers, including glioblastoma. EGFR amplification is noted mostly in primary GBMs (~60%) and rarely in secondary GBMs (~10%). EGFRvIII (variant III), the most frequent mutation in the overexpression of EGFR, is exclusively detected in cancer cells and strongly associated with an unfavorable survival prognosis[20]. Therefore, studies have been targeting EGFRvIII for therapeutic purposes, and some progress has been made with the vaccine Rindopepimut in clinical trials[21, 22].

Other molecular markers that also present prognostic value are glioma-CpG island methylator phenotype (G-CIMP), ATRX mutation, TERT mutation, and genetic losses of chromosomes[23].

1.1.3 Clinical symptoms and diagnosis

The clinical manifestations of glioblastomas are usually dependent on the size, location, and growth rate of the tumor. Common symptoms include headache, seizure, nausea or vomiting, memory loss, personality change, and focal neurological deficits[24]. Most GBM patients present a short clinical history ranging from 3 to 6 months[25].

The typical non-invasive examination tool for a presumptive diagnosis is magnetic resonance imaging(MRI) or computed tomography(CT). MRI is the current gold standard for the GBM imaging technique. The imaging feature presents an infiltrative, heterogeneous, ring-enhancing mass lesion with necrotic central areas and extensive peritumoral edema[26]. Contrast-enhanced MRI, magnetic resonance spectroscopy (MRS) and positron emission tomography (PET) may provide additional valuable

information about the tumor in certain cases[27, 28]. For a confirmatory diagnosis, it requires a histopathological analysis of a tumor tissue obtained by surgical resection or stereotactic biopsy.

1.1.4 Treatment and prognosis

The current standard care for newly diagnosed GBM is the combined treatment of surgical resection, radiotherapy and chemotherapy. Surgery has always been a primary component of GBM care, as a greater extension of resection improves overall survival[29]. However, surgery alone leads to only 3-6 months of median survival time[30], which is why the following adjuvant chemotherapy and radiotherapy are imperative. The alkylating agent TMZ is the first-line chemotherapy drug for GBM treatment. A phase III clinical trial confirmed that radiotherapy plus temozolomide chemotherapy had an advantage of prolonged survival over radiation alone[31].

With an increasing understanding of the heterogeneity and pathogenesis of GBM, researchers were able to explore novel approaches to treat this highly aggressive cancer, trying to improve its prognosis. The distinct immune environment of the central nervous system and the complex immunosuppressive property of the glioblastoma microenvironment have led to extensive research of immunotherapy, which currently includes vaccination therapy, immune-checkpoint blockade, oncolytic viral therapy and chimeric antigen receptor T cell therapy[32]. Another major part is the targeted therapy, which is based on the identification of various molecular biomarkers and molecular pathogenesis that influence tumor development. For example, inhibitors that target cellular signaling pathways (PI3K/Akt/mTOR, p53 and retinoblastoma pathways) and receptor tyrosine kinases (EphA3, VEGF, PDGFR and MET)[33].

Enormous efforts have been devoted to optimizing standard treatment and developing novel targeted strategies, which have contributed to the aim of personalized therapy for glioblastoma patients. However, the prognosis of GBM patients still remains very

poor, with a median survival of 12-15 months[31, 34], a five-year survival of 6.8%[4] and a ten-year survival of 0.71%[35]. The path of uncovering better therapeutic strategies for GBM treatment remains challenging yet necessary.

1.2 The role of inflammation in glioblastoma

Inflammation is a host defensive process initiated by innate immunity in response to certain stimuli, such as pathogens, injuries or foreign objects. Innate immunity activates antigen presentations, as well as numerous inflammatory cytokines and mediators that recruit more immune cells and induce adaptive immunity activation[36]. Adaptive immunity can further amplify the inflammation, while some regulatory immune cells negatively control the inflammatory response, such as regulatory T cells (Tregs)[37]. Effective immunity can only be achieved when all immune cells and immunoreactions function in harmony with appropriate immunosuppressive mechanisms. However, when the inflammatory immune response is not adequately regulated and terminated, the chronic inflammation settles. Although inflammation is designed for host defense, chronic inflammation is usually considered detrimental[38]. Particularly, inflammation has been viewed as one of the hallmarks of cancer[39]. Ever since Rudolf Virchow’s discovery of leukocytes within tumors in 1863, which firstly indicated a connection between inflammation and cancer, mounting evidence over the decades has not only confirmed the decisive role of inflammation in tumorigenesis, but also elucidated some of the cellular and molecular mechanisms.

1.2.1 The inflammatory GBM microenvironment

A tumor is not merely a mass of malignant cells. The glioblastoma tumor microenvironment (TME) is characterized by its extensive heterogeneity. GBM cells and glioma stem cells are embedded with multiple parenchymal cells, including vascular cells, various immune cells and other glial cells[40]. The tumor-immune cell

interplay generates an abundance of inflammatory mediators and results in chronic inflammation that promotes tumor progression[41]. Inflammation facilitates every step of tumorigenesis, including tumor initiation, tumor promotion, angiogenesis and metastasis[2]. In the meantime, the tumor enhances the inflammatory response by producing chemokines and cytokines, which will recruit and mediate immune cells and further deteriorate the inflammatory TME. The mutually reinforcing interaction of tumor and inflammation aggravates tumor malignancy[42]. Therefore, investigating the intricate network of cell communications and molecular signaling pathways in the inflammatory TME is of great significance for GBM research.

1.2.2 The hypoxic GBM microenvironment

Hypoxia is a prominent feature in the TME, especially in the rapidly growing glioblastoma. Hypoxic regions always occur in solid tumors, mostly due to uncontrolled tumor cell proliferation and imperfect neovascularization[43]. Hypoxia induces the expression of a transcription factor, hypoxia-inducible factor 1 (HIF1), which functions in regulating the hypoxia responsive genes and facilitating tumor development and therapy resistance[44]. The most crucial angiogenic mediator, vascular endothelial growth factor (VEGF), can be induced by hypoxia via HIF1 activation in GBM, resulting in promoted angiogenesis[45]. However, the hypoxia-induced angiogenic response mostly generates aberrant tumor vasculature with inadequate oxygen-diffusing function due to abnormal vessel morphology, excessive branching, thrombotic occlusion, arteriovenous shunts and low structural integrity, which further exacerbate the hypoxia level and in turn stimulate more HIF1 activation[46]. Thus, a vicious hypoxia cycle is formed in the tumor microenvironment. Additionally, it has been demonstrated that hypoxia and the HIF1 pathway in GBM accelerate tumor cell proliferation, invasion and metastasis[47, 48], and also enhance tumor resistance to chemotherapy and radiotherapy[44]. Of note, hypoxia induces the activation of NF-κB and various pro-inflammatory cytokine expressions, such as IL-6, IL-8, COX2 and CCL2, leading to an enhanced and prolonged cancer-related inflammation[49-52]. In

this way, hypoxia and inflammation, as the two predominant hallmarks of GBM, intertwine and aggravate tumor aggressiveness.

1.2.3 IL-1 signaling and IL-1β-induced cytokines in the GBM microenvironment

The interleukin-1 (IL-1) family comprises two major agonists, IL-1α and IL-1β, which both bind to the IL-1 receptor and activate the same biological functions. IL-1α is mostly active in its cytosolic precursor form pro-IL-1α or the membrane-bound form, whereas IL-1β only acquires its bioactive functions in the secreted form in response to inflammatory stimuli[53]. IL-1α can act as an alarmin to initiate sterile inflammation, which induces the necrosis of cells along with IL-1α release[54]. However, within the tumor milieu, extracellular IL-1α level is usually too low to activate extensive inflammation that accelerates tumor progression. On the other hand, IL-1β produced by both tumor cells and immune cells exists abundantly in the GBM microenvironment and exacerbates the tumor-associated inflammation[55].

IL-1β is regarded as a master cytokine that amplifies the inflammatory cascade and is involved in multiple malignant processes in many types of cancer, typically in glioblastoma. Elevated expression of IL-1β has been observed in some GBM cell lines[56], as well as in human GBM tissue specimens[57]. IL-1β binds to the IL-1R and activates the transcription factor NF-κB and the p38 MAPK signaling pathway, resulting in an upregulation of various downstream target genes[58, 59]. Serving as a mechanistic link between inflammation and tumorigenesis, NF-κB signaling has been associated with a series of tumor-promoting effects – acceleration of tumor proliferation, metastasis and angiogenesis, and inhibition of tumor cell apoptosis[60]. Under normoxic conditions, activation of p38 MAPK and JNK signaling upregulates VEGF secretion from GBM cells, inducing VEGF-mediated angiogenesis[61]. Moreover, IL-1β can boost high level productions of not only IL-1β itself in GBM, but also various other pro-inflammatory cytokines, such as IL-6, IL-8, CCL2 and COX2,

which further activate their downstream signaling pathways and exacerbate the tumor-promoting inflammation[62-64].

Interleukin-6 (IL-6) is secreted by various types of cells, including malignant cells, in response to external stimuli such as tissue damage, stress or the stimulation of other cytokines (IL-1β and TNFα). A higher gene expression of IL-6 was found in glioblastoma tissues compared to other lower grade gliomas[65], suggesting a vital role in GBM malignancy. IL-6 signaling pathways activate the JAK proteins, which lead to the phosphorylation of the downstream STAT transcription factors, particularly signal transducer and activator of transcription-3 (STAT3)[66]. STAT3 signaling pathway participates in mediating cancer inflammation[67]. Overexpression of STAT3 was found in human gliomas and correlated with immune cell infiltration and pathological tumor grading[68]. Studies have demonstrated that IL-6 promotes glioblastoma invasion and angiogenesis via JAK/STAT3 signaling[69, 70]. In a variety of cancers, STAT3 activation can induce the expression of a number of anti-apoptotic markers, such as Bcl-2, Bcl-X and mcl-1[71]. Inhibition of STAT3 signaling results in a reduction of the Bcl-2 family proteins, generating a less aggressive GBM phenotype with suppressed proliferation and promoted apoptosis[72].

Interleukin-8 (IL-8) is another key inflammatory mediator that possesses a potent pro-angiogenic function in the context of tumor. Cytokine stimulation of IL-1 or TNFα, and macrophage infiltration was found to enhance IL-8 expression in GBM cells, and anti-inflammatory agents were able to suppress this upregulation[73]. In addition, a meta-analysis has revealed that a number of circulating inflammatory factors, including IL-6 and IL-8, are associated with increased glioma risk and a poor prognosis in glioma patients[74]. IL-8 mRNA expression in glioma surgical specimens was found positively correlated with the microvessel count and infiltrating tumor-associated macrophages (TAM)[73]. NF-κB signaling regulates IL-8 expression and contributes to tumor angiogenesis[75]. Activation of the transcription factors, NF-κB and activator protein 1

(AP-1), promotes GBM migration and invasion via IL-8 regulation[76].

The C-C motif chemokine ligand 2 (CCL2), also known as monocyte chemoattractant protein-1(MCP-1), is a small cytokine member of the CC chemokine family. CCL2 functions by binding to its receptor CCR2, which is expressed in multiple types of cells, including malignant cells, endothelial cells, monocytes and dendritic cells[77]. As a potent chemoattractant, CCL2 mediates multiple inflammatory responses and pro-tumorigenic activities by recruiting immune cells to the tumor sites[78-80]. Higher CCL2 level was found in malignant gliomas and in correlation of macrophage infiltration[81]. CCL2, along with IL-6, facilitates myeloid monocyte recruitment and their differentiation towards M2-type macrophages with tumor-promoting features[82]. Within the glioblastoma microenvironment, CCL2 induces the accumulation of immunosuppressive Tregs and myeloid-derived suppressor cells (MDSC)[83], counteracting the effective anti-tumorigenic immune response. CCL2 is also one of the critical regulators of T cell differentiation, inducing the differentiation of regulatory T cells[84, 85]. Moreover, GBM-secreted CCL2 was able to stimulate CCR2-bearing microglia to produce IL-6, which in turn promoted GBM invasiveness[86].

Cyclooxygenase-2 (COX2) is an enzyme that catalyzes arachidonic acid to prostaglandin endoperoxide H2 (PGH2), which further converts to five primary prostanoids, including prostaglandin D2 (PGD_2), prostaglandin E2 (PGE_2), prostaglandin F2α ($PGF_{2\alpha}$), prostacyclin (PGI2) and thromboxane A2 (TXA2)[87]. COX2 has an isozyme COX1, which is constitutively expressed in most tissues, regulating a number of prostanoid-related physiological functions[88]. Contrary to COX1, COX2 expression in normal tissue cells is very low, but an elevated expression level is often induced by inflammation. In line with the concept of tumor-associated inflammation, mounting evidence has revealed the high level of COX2 and its pro-tumoral effects in a variety of cancers[89]. In glioblastoma studies, COX2 expression is positively correlated with GBM pathological grade and poor prognosis[90, 91]. COX2

engages in many aspects of GBM aggressiveness, including proliferation, invasion, apoptosis, angiogenesis, immunosuppression and therapy resistance, predominantly through the downstream product PGE_2 binding to its receptors[92]. Inhibitors targeting either COX2 or PGE_2 have presented anti-tumor effects[89, 93].

1.3 Immune cells within the GBM microenvironment

The immune cells in the TME mediate the balance of anti-tumor immunity and pro-tumorigenic inflammation. However, this balance is more likely to be protumoral, as tumors tend to grow continuously and vigorously when no therapeutic interventions are applied. Among the multiple kinds of immune cells that function in the inflammatory tumor microenvironment, T cells and TAMs are of particular significance in regulating the tumor-promoting inflammation[2].

1.3.1 T cells

The T cell population is generally classified into a series of subsets according to their different functions: cytotoxic CD8+ T cells and helper CD4+ T cells. As a major component of adaptive immunity, T cells are indispensable for anti-tumor immune response. It is reported that increased T cell infiltration in tumors results in a more favorable outcome in multiple cancers, such as breast cancer, lung cancer and gastric cancer[94-96]. However, the T cell functions vary among the different subsets in the context of tumor. Evidence has shown that T cells are also involved in tumor growth, metastasis and progression. The infiltrating T cells and their cytokine profile exert a dual effect on GBM cells, participating in both adaptive anti-tumor immunity and tumor-promoting functions.

1.3.1.1 T cell subsets

CD8+ T cells are the most prominent immune cells for killing cancer in adaptive

immunity. By recognizing the histocompatibility complex (MHC) class I molecules on the antigen-presenting cells (APCs), naïve CD8+ T cells get activated to become cytotoxic T lymphocytes (CTLs), acquiring their effector functions. CTLs kill tumor cells by releasing the cytotoxins, mainly perforin 1 and granzyme B, which enter into the target tumor cells, trigger the caspase cascade, and eventually cause cell apoptosis. Perforin 1 binds to the membrane of the target tumor cell and forms transmembrane pores, allowing the passive diffusion of granzyme B into the tumor cell. Granzyme B is a serine protease that initiates apoptosis of tumor cells by activating the caspase signaling[97, 98]. Expressions of perforin and granzyme B have been associated with effective anti-tumor immunity[99, 100]. Another way to induce apoptosis is through the direct Fas-Fas ligand interaction on the cell surface. The Fas ligand on CTLs can bind to the Fas molecules on the tumor cells, resulting in apoptotic death. A high density of cytotoxic CD8+ T cell infiltration at initial presentation was found to be associated with good clinical prognosis and prolonged survival in GBM[101].

Helper CD4+ T cell cells express antigen receptors that recognize the MHC class II molecules. Once activated by antigens, helper CD4+ T cells exert their adaptive immunity by the robust production of chemokines and cytokines. In order to generate specific immune responses, helper T cells differentiate into a variety of T helper (Th) subsets. The most commonly recognized Th subsets are Th1, Th2, Th17 and Tregs[102].

Th1 cells are also generally considered to exert a favorable anti-tumor immune response. Th1 cells feature in secreting abundant pro-inflammatory effector cytokines, including interferon γ (IFNγ) and TNFα. These cells can not only promote and regulate the anti-tumor activity of CTLs, macrophages and natural killer (NK) cells, but also induce antigen presentation of tumor cells. Th2-cell-produced cytokines, IL-4 and IL10, are reported to be associated with tumor clearance and angiogenesis inhibition[103, 104]. However, some studies demonstrated that Th2 cytokines downregulate anti-

tumor CTL activity[105, 106]. A predominant expression of Th2 cytokines was found in human gliomas[107]. Th1/Th2 cytokine balance is regarded as an essential factor for GBM prognosis[108]. Low Th2 balance is associated with a better prognosis in GBM[109].

Th17 cells have been found to play a paradoxical and complex role in tumor immunity. Th17 differentiates from naïve CD4+ T cells in response to IL-6, TGF-β and IL-1β through the activation of STAT3 signaling[110]. Th17 cells predominantly secret cytokines as IL-17, IL-21 and IL-22, which enable a dual function of Th17 – tumor suppression by increasing immune cell recruitment; and pro-tumor effect by inducing Treg infiltration and promoting tumor cell proliferation, progression and metastasis[111]. Moreover, unlike other stable lineages of T cell subsets, Th17 cells present a high potential of plasticity. They can transdifferentiate into other types of Th cells, mostly Th1 or Tregs, exerting their corresponding anti-tumor or pro-tumor effector functions[112]. In GBM patients, high infiltration of Th17 cells is related to a poor prognosis[113]. Th17 cells in the GBM microenvironment may induce immune suppression via IL-10 secretion[114].

Tregs are known to have immunosuppressive features, which not only regulate the excessive immune response to self-antigens, but also can suppress the effective anti-tumor immunity in TME. Tregs function via the production of immunosuppressive cytokines, IL-10 and transforming growth factor β (TGF-β)[102]. In the TME, Tregs suppress the immune activity of other T cells by impairing their cell proliferation and cytokine production[115]. An increase of CD4+CD25+FOXP3+ Tregs was observed in GBM patients, exhibiting immunosuppressive activity[116].

1.3.1.2 T-cell-secreted cytokines

IFN-γ is the only type II interferon cytokine that is predominantly secreted by NK cells and T cells, as a part of the innate and adaptive immune response[117]. The role of

IFN-γ in tumor immunity is paradoxical. IFN-γ supports tumor immune surveillance by upregulating the antigen-presenting MHC expression on tumor cells[118, 119]. IFN-γ also plays a vital role in the recruitment, differentiation and anti-tumor immune response of T cells and NK cells[120, 121]. Moreover, IFN-γ induces cancer cell apoptosis[122] and indirectly serves as an antiangiogenic factor[123]. However, in the clinical trials for GBM treatment, IFN-γ treatment did not show an improved prognosis compared to the control group[124, 125], indicating the limited anti-tumor effect of IFN-γ in glioblastoma. Of note, accumulating evidence has revealed a "dark side" of IFN-γ. IFN-γ can not only promote tumor growth, but also facilitate tumor immune evasion by upregulating the immune checkpoints. Besides, it induces an immunosuppressive tumor microenvironment by enhancing the immunoregulatory enzyme IDO secretion from tumor cells[126]. Particularly, IFN-γ has been demonstrated to be a major cause for the immune checkpoint programmed death-ligand 1 (PD-L1) expression in glioblastoma[127], which is essential in mediating tumor immune escape[128]. Therefore, the role of IFN-γ in the context of GBM is more likely to be tumor-promoting.

IL-17 is a potent pro-inflammatory cytokine that is mainly produced by Th17 cells. IL-17 binds to its receptor IL17R and triggers the activation of three major transcription factors and their signaling pathways, including NF-κB, MAPK and C/EBP[129]. IL-17 targets on a variety of cells to stimulate the secretion of abundant pro-inflammatory cytokines, such as IL-1β, TNFα, IL-6, IL-8 and PGE_2, amplifying the inflammatory response[130, 131]. It is reported that IL-17 induces IL-6 and IL-8 production in GBM cell lines[132]. Upregulated expression of IL-17 in U87 GBM cells enhances tumor growth[133]. IL-17 manifests tumor-promoting functions by activating PI3K/Akt signaling in GBM cells and facilitates tumor migration and invasion[134].

IL-22 is a pro-inflammatory cytokine that belongs to the IL-10 family and is produced by immune cells, especially activated T cells such as Th1, Th17 and Th22 cells. Unlike most other cytokines, IL-22 does not trigger inflammatory responses in immune cells,

due to the fact that its receptor IL-22R is absent on immune cells but only expressed on epithelial cells, fibroblasts and other tissue cells, including tumor cells[135]. IL-22 signaling involves the phosphorylation and activation of the JAK/STAT3 pathway, generating a series of tissue-protective and repairing effects[136]. Dysregulation of IL-22 results in several autoimmune diseases, such as rheumatoid arthritis[137]. IL-22 promotes the proliferation, chemotaxis and neovascularization of epithelial cells, contributing to tumor angiogenesis[138]. The tumor-promoting role of IL-22 has been elucidated in glioblastoma and a number of other cancers[139-141]. GBM cell lines only express IL-22R but not IL-22. Exogenous IL-22 induces cell proliferation and anti-apoptotic effect on GBM through STAT3, PI3K/Akt and ERK1/2 pathways[142], suggesting the IL-22/IL-22R signaling is activated by the inflammatory tumor microenvironment. In a murine glioblastoma study, IL-22 boosted the cytokine production of IL-1β, IL-6 and TNFα. Moreover, IL-22 inhibiting measurements, IL-22 knock-out mice and IL-22-neutralising antibody treatment have demonstrated protective effects with prolonged survival and decreased cytokine levels[143].

IL-10 is an anti-inflammatory and immunosuppressive cytokine produced by various types of immune cells and tumor cells. IL-10 strongly suppresses the production of numerous pro-inflammatory cytokines such as IL-1, IL-6 and IL-8, and inhibits the antigen presentation of APCs[144, 145]. In regard to tumor immunity, the role of IL-10 remains controversial. Elevated IL-10 expression was found in the tumor tissue and serum of glioma patients and positively correlated with tumor grade, cell proliferation and migration[146]. GBM-cell-derived IL-10 suppresses antitumor response by decreasing cytokine production of immune cells and by inhibiting T cell proliferation and cytotoxicity[147]. On the other side, T-cell-produced IL-10 mediates tumor rejection. It is reported that IL-10 inhibits the immunosuppressive CD4+ T cells and enhances antitumoral CTL persistence in a plasmacytoma model[148]. In another glioma mouse model study, IL-10-producing CD4+ T cells have exhibited protective anti-tumor properties to accelerate tumor rejection[149].

1.3.2 Tumor-associated macrophages

TAMs are derived from circulating monocytes or tissue-resident macrophages infiltrated in the tumor site[150]. TAM activation results in two subtypes: the classical pro-inflammatory M1 phenotype and the alternative anti-inflammatory M2 phenotype[151]. TAMs are vital players in the TME that contribute to tumor-associated inflammation and facilitate tumor development. The tumor-promoting functions of TAMs include angiogenesis, immunosuppression, tumor growth, metastasis, and therapy failure[152-154]. High TAM infiltration generally correlates with unfavorable outcomes in malignancies, such as breast cancer and lung cancer[155, 156]. TAM-secreted cytokines, such as IL-6 and CCL8, have been associated with GBM progression[157, 158]. In GBM patients, M1 phenotype expression was found to have a negative correlation with pathological grades, while M2 type level was positively correlated with glioma grades, indicating that the M2 ratio may serve as a negative prognostic factor in gliomas[159].

1.4 The recombinant IL-1 receptor antagonist – Anakinra

Anakinra (Kineret®) is a recombinant human IL-1 receptor antagonist, blocking the biological activity of IL-1α and IL-1β, thereby attenuating IL-1-mediated inflammatory signaling. It was firstly approved by the Food and Drug Administration (FDA) in 2001 for the treatment of rheumatoid arthritis(RA)[160]. Over the years, Anakinra has been applied for treating various autoinflammatory diseases and also broadly been investigated for other IL-1-involved conditions.

As IL-1 is recognized as a major orchestrator of chronic inflammation that drives tumor progression, IL-1 blockade has been proposed as a novel therapeutic candidate for human cancer treatment[161]. It was firstly reported in 1993 that IL-1Ra inhibited IL-1-induced metastasis of human melanoma in mice[162]. A study on hepatic metastasis of melanoma also supported this finding and provided additional information that even

a single dose of the short half-life IL-1Ra was able to reduce the metastasis by 50%, and the 10 daily doses resulted in an 80% reduction[163]. VEGF secretion is induced by IL-1β and significantly decreased by IL-1Ra in colorectal carcinoma cell lines and tumors, implicating an antiangiogenic effect of IL-1Ra[164]. In breast cancer, anakinra was reported to mitigate tumor proliferation, bone metastasis and angiogenesis[165]. It is also assumed that anakinra suppresses pancreatic cancer progression by abrogating NF-κB activity[166]. To date, only a few studies have applied IL-1Ra in glioblastoma treatment. IL-1Ra inhibits IL-1β-promoted proliferation, migration and invasion in GBM cell lines[167]. In C6 glioma rats, IL-1Ra administration significantly reduces peritumoral edema and prolongs their life span in a dose-dependent manner[168]. These studies have suggested IL-1Ra might have a beneficial efficacy in treating glioblastoma. However, no previous research has elucidated the impact of IL-1Ra on the inflammatory crosstalk between GBM cells and immune cells in the tumor microenvironment.

Regarding the current clinical use for anakinra, a subcutaneous injection of 100mg per day is recommended for RA patients. After administration, it takes about 3-7 hours to reach the maximum plasma concentration with a half-life of 4-6 hours[160]. Multiple clinical trials have provided the safety profile of anakinra. It is reported that anakinra did not significantly increase the total adverse events compared to the placebo (90.0-93.8% vs. 81.0-93.4%). The most common adverse event was the dose-related injection site reaction, which occurred significantly higher in the anakinra group than the placebo(19-81% vs. 24.0-29.6%)[169]. However, it is usually mild and resolves within 2-3 weeks. A meta-analysis has concluded that anakinra induced an increased risk of infection in a dose-dependent manner[170]. But the total rate of infections was not significantly elevated by anakinra compared with placebo (5-40% vs. 12.0-45.4%)[169]. Other adverse effects include headache, diarrhea, malignancies, hematologic events, immunogenicity and laboratory abnormalities, most of which anakinra does not result in much aggravated outcomes[160]. Even in pediatric patients,

anakinra was well tolerated and displayed a similar safety profile as in adults[171].

As a 17.3kD protein with 153 amino acids, anakinra is able to penetrate the blood-brain barrier (BBB) and functions in the CNS. After intravenous administration, anakinra concentration in the cerebrospinal fluid (CSF) peaked 1-2h after bolus doses and declined with a half-life of 4.7h, slower than in serum (2.9h)[172]. It has been revealed that anakinra can alleviate the CNS symptoms in patients with neonatal-onset multisystem inflammatory disease (NOMID)[173, 174]. Other animal studies have reported favorable outcomes with anakinra administration in some cerebral inflammatory conditions, such as cerebral ischemia[175], traumatic brain injury[176] and seizures[177]. Subcutaneous administration of anakinra requires very high doses to exert neuroprotective effects in rats[178]. Another study suggested that intravenous anakinra administration of 100mg bolus followed by 2mg/kg/h infusion for 24h in patients resulted in CSF concentrations that were comparable to the neuroprotective concentrations in rats[179].

Other IL-1 inhibitors include rilonacept and canakinumab. Rilonacept is a soluble dimeric fusion protein with a molecular mass of 251kD and a half-life of 67h. Canakinumab is a human anti-IL-1β monoclonal antibody with a molecular weight of 145.2kD and a half-life of 21-28 days[180]. They both have a much longer half-life than anakinra, which could reduce the administration frequency and effectively resolve the most frequent adverse event of anakinra, the injection site reactions. However, the molecular weights of these two inhibitors are much larger, which may prevent them from crossing the BBB. In a clinical trial comparing anakinra and canakinumab in treating NOMID patients, the inflammatory markers in CSF were significantly higher in canakinumab-treated patients than in anakinra, whereas their levels in the serum showed no difference, indicating that anakinra has better efficacy in suppressing CNS inflammation[181]. Therefore, rilonacept and canakinumab may not be suitable for treating intracranial diseases, such as brain tumors.

1.5 Hypothesis and aims of the study

Glioblastoma is one of the most devastating cancers with an abysmal prognosis. The intricate crosstalk of tumor cells and immune cells induces an inflammatory microenvironment in GBM, which accelerates tumor development. IL-1β has been demonstrated to orchestrate inflammatory signaling in both GBM cells and immune cells. Therefore, we hypothesized that the IL-1β plays a decisive role in mediating the pro-inflammatory signaling in the GBM microenvironment that drives tumor progression. By blocking IL-1β signaling, the recombinant IL-1Ra anakinra might ameliorate the vicious cycle of self-aggravating inflammation and reduce GBM aggressiveness. Thus, this present study could provide an experimental basis for a novel therapeutic approach for GBM treatment.

To date, the intricate network between GBM and immune cells in the inflammatory microenvironment has not been fully elucidated. Therefore, we aimed to investigate how the GBM-immune cell interplay affects cytokine expression and phenotype changes, and whether anakinra has a beneficial impact on this GBM-immune cell network. To this end, the following objectives were addressed in this study:

1) To investigate the role of IL-1β within the inflammatory GBM microenvironment - whether the IL-1β-induced inflammation will lead to a more aggressive GBM phenotype.
2) To elucidate the inflammatory signaling pathways within the intricate network consisting of GBM and immune cells in the TME. An in vitro co-culture model that mimics the GBM-immune cell interplay was to be established.
3) To examine the impact of anakinra on IL-1β-induced inflammation and tumor-immune cell inflammatory crosstalk in the GBM environment - whether anakinra could ameliorate tumor-associated inflammation in the GBM environment by blocking IL-1 signaling, and thereby attenuate GBM aggressiveness.

2. Materials and methods

2.1 Materials

2.1.1 Laboratory Equipments

Laminar flow workbench	Weiss Technik, Germany
Cell culture incubator	Thermo Fisher Scientific, USA
Centrifuge, Rotina 35R	Hettich, Germany
Centrifuge, Heraeus Megafuge 40R	Thermo Fisher Scientific, USA
Microfuge, 5424	Eppendorf AG, Germany
Microfuge, 5451 R	Eppendorf AG, Germany
Shaking water bath, SWB25	Thermo Haake, USA
Digital Vortex Mixer	VWR, USA
4°C fridge	LIEBHERR, Germany
-20°C fridge	Privileg, Germany
-80°C fridge	Heraeus Holding GmbH, Germany
Incubator chamber for hypoxia	Billups-Rothenberg, USA
Vi-CELL	Beckman-Coulter, USA
Nalgene Mr. Frosty Freezing Container	Thermo Fisher Scientific, USA
gentleMACS Dissociator	Miltenyi Biotech GmbH, Germany
AutoMACS Pro Separator	Miltenyi Biotech GmbH, Germany
Nanodrop 2000 spectrophotometer	Thermo Fisher Scientific, USA
Thermo-Shaker, PCMT	Grant Instruments, UK
Thermocycler, Mastercycler gradient	Eppendorf AG, Germany
LightCycler 480	Roche Diagnostics GmbH, Germany
Flow cytometer, BD FACSCanto II	BD, USA
Inverted microscope	Zeiss, Germany
Stage Top Incubation System	Ibidi GmbH, Germany

FilterMax F3 MultiMode Microplate Reader	Molecular Devices, Germany
Electrophoresis Chamber	Bio-Rad, USA
Western Blotting Transfer Systems	Bio-Rad, USA
Standard power pack P25	Biometra GmbH, Germany
Digital imaging system	Hamamatsu Photonics, Japan
Orbital shaker	Heidolph Instruments, Germany
Plate shaker	NeoLab, Germany
Micropipettes	Eppendorf AG, Germany
Pipette Controller	Integra Biosciences AG, Switzerland

2.1.2 GBM cell line

T98G	ACTT, USA

2.1.3 Cell culture medium and supplements

DMEM	Gibco, USA
RPMI-1640	Gibco, USA
MACS Neuro medium	Miltenyi Biotech GmbH, Germany
FCS	Biochrom GmbH, Germany
HEPES	Gibco, USA
L-Glutamine	Bio&Sell, Germany
NEAA	Gibco, USA
Penicillin/streptomycin	Gibco, USA
Neuro Brew-21 without vitamin A	Miltenyi Biotech GmbH, Germany

2.1.4 Chemical reagents

IL-1β	Miltenyi Biotech GmbH, Germany
Anakinra	Swedish Orphan Biovitrum AB, Sweden

Trypsin 10X	Lonza Bioscience, Switzerland
Accutase	Sigma-Aldrich, USA
PBS	University hospital LMU, Germany
DMSO	Sigma-Aldrich, USA
HBSS	University hospital LMU, Germany
Histopaque-1077	Sigma-Aldrich, USA
Dynabeads Human T-Activator CD3/CD28	Thermo Fisher Scientific, USA
Primers	Metabion, Germany
RealTime Ready Single Assay	Roche Diagnostics GmbH, Germany
UPL probes	Roche Diagnostics GmbH, Germany
FastStart Essential DNA Probes Master	Roche Diagnostics GmbH, Germany
Albumin Fraction V	CARL ROTH, Germany
Ethanol absolute	VWR, USA
10X Cell Lysis Buffer	Cell Signaling, USA
Protease inhibitor	Cell Signaling, USA
Phosphatase inhibitor	Cell Signaling, USA
Nonfat dry milk	Bio-Rad, USA
30% Acrylamide	Sigma-Aldrich, USA
APS	Bio-Rad, USA
TEMED	Bio-Rad, USA
SDS	Roche Diagnostics GmbH, Germany
Tris	Sigma-Aldrich, USA
Glycine	Sigma-Aldrich, USA
NaCl	Sigma-Aldrich, USA
Glycerol	Sigma-Aldrich, USA
2-Mercaptoethanol	Sigma-Aldrich, USA
Bromophenol blue	Thermo Fisher Scientific, USA
Tween-20	Sigma-Aldrich, USA

2.1.5 Commercial kits

Brain Tumor Dissociation Kit	Miltenyi Biotech GmbH, Germany
Pan T Cell Isolation Kit	Miltenyi Biotech GmbH, Germany
RNAqueous™ Total RNA Isolation Kit	Ambion, USA
TURBO DNA-free kit	Invitrogen, USA
cDNA synthesis kit	Invitrogen, USA
Violet Chromatin Condensation/Dead Cell Apoptosis Kit	Invitrogen, USA
CytoSelect Collagen Cell Invasion Assay Kit	Cell Biolabs, USA
LEGEND MAX™ Human ELISA Kit	BioLegend, USA
ELISA MAX™ Deluxe Set	BioLegend, USA
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific, USA
Clarity^{TM} Western ECL Substrate kit	Bio-Rad, USA

2.1.6 Antibodies

Alexa Fluor 488 anti-human Ki-67 antibody	BioLegend, USA
Alexa Fluor 488 Mouse IgG1, κ antibody	BioLegend, USA
Bcl-2 (D55G8) Rabbit mAb	Cell Signaling, USA
β-Actin (13E5) Rabbit mAb	Cell Signaling, USA
Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling, USA

2.1.7 Disposable materials

Micro tube 1.5ml	Sarstedt AG&Co, Germany
Micro tube 2ml	Sarstedt AG&Co, Germany
DNase, RNase free tube 1.6ml, green	Biozym Scientific, Germany
DNase, RNase free tube 1.5ml	Biozym Scientific, Germany
DNase, RNase free tube 2ml	Biozym Scientific, Germany
Safe-Lock tubes 0.5ml	Eppendorf AG, Germany

Cell culture flasks, standard growth surface, red cap	Greiner Bio-One GmbH, Germany
Cell culture flasks, Cell+ growth surface, yellow cap	Sarstedt AG&Co, Germany
6-well plates	Greiner Bio-One GmbH, Germany
12-well plates	Greiner Bio-One GmbH, Germany
24-well plates	Greiner Bio-One GmbH, Germany
96-well plates for PCR, white	Roche Diagnostics GmbH, Germany
96-well assay plates, clear	Corning Inc., USA
Cell scrapers	Sarstedt AG&Co, Germany
CryoTube Vials	Thermo Fisher Scientific, USA
Vi-CELL sample cups	Beckman-Coulter, USA
ThinCert™ cell culture inserts, 0.4µm translucent	Greiner Bio-One GmbH, Germany
gentleMACS C Tubes	Miltenyi Biotech GmbH, Germany
50ml tubes	Greiner Bio-One GmbH, Germany
50ml tubes with filter	Greiner Bio-One GmbH, Germany
15ml tubes	Sarstedt AG&Co, Germany
FACS tubes, 5ml	Sarstedt AG&Co, Germany
Pipette tips	Eppendorf AG, Germany
SafeSeal Tips 200µl	Biozym Scientific, Germany
SafeSeal Tips 1000µl	Biozym Scientific, Germany
Filter Tips 10µl	Sarstedt AG&Co, Germany
Filter Tips 20µl	Sarstedt AG&Co, Germany
Serological pipettes	Sarstedt AG&Co, Germany
µ-Slide Chemotaxis	Ibidi GmbH, Germany

2.2 Methods

2.2.1 Cell culture

All cells were cultured in a humidified cell culture incubator at 37°C and 5% CO_2. All

the handling of cells was operated under a laminar flow cabinet to avoid contamination. Glioblastoma cell lines were cultivated in culture flasks with red vented caps and standard growth surface for adherent cells. Primary GBM cells were cultured in yellow cap culture flasks with Cell+ growth surface for sensitive adherent cells. The size of culture flasks was determined by how many cells were needed for the following experiment setup. Cell culture media and supplements were stored in 4°C or -20°C fridges and were warmed up to 37°C in a water bath before applied to cells.

2.2.1.1 GBM cell line

T98G cells were grown in 75cm^2 cell culture flasks with approximately 10ml of culture medium - Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acids (NEAA), and 1% penicillin/streptomycin. Cells were harvested and split when they had reached 80-95% confluence. All experiments were conducted using cells within ten passages. The subculturing process is as follows:

- Remove the culture medium from the flask.
- Incubate the cell layer with 5ml 1x trypsin/EDTA solution in the incubator for 5 min.
- Gently flap the sidewall of the flask several times to detach the cells.
- Inactivate the trypsin and wash off the cells with 10ml FCS containing medium.
- Transfer the cell suspension to a new 50ml falcon and centrifuge for 5min at 1700rpm, room temperature.
- Discard the supernatant and gently resuspend the cell pellet in 1ml culture medium.
- Prepare a Vi-CELL sample cup with 480ul phosphate-buffered saline (PBS) in it. Add 20ul of cell suspension and count the cell number using a Vi-CELL analyzer.
- Record the cell viability and viable cell number provided by the Vi-CELL.
- Seed an appropriate number of cells in a new culture flask with fresh DMEM culture medium.

2.2.1.2 Primary GBM cell culture

Primary GBM cells were obtained from clinical patients undergoing open tumor resection. The study was approved by the local ethics committee of the Ludwig-Maximiliams-University Munich (approval no.216/14). Tumor tissues were processed using Brain Tumor Dissociation Kit(P) and a gentleMACS Dissociator. Tissues were cut into smaller fragments under sterile conditions before transferred into gentleMACS C Tubes containing preheated buffer X. The following steps were performed according to the manufacturer's protocol. In brief, Enzyme N, Buffer Y, and Enzyme A were added subsequently to the tube. Dissociation programs h_tumor_02, h_tumor_03 and m_brain_01 were performed successively using the gentleMACS Dissociator. After dissociation, the cells were cultured in yellow cap flasks (Cell+ growth surface) with MACS Neuro Medium supplemented with 10% FCS, 2% L-Glutamine, 2% penicillin/streptomycin, and 2% Neuro Brew-21 without vitamin A and were labeled as the passage 0. After 2-4 days, GBM cells would adhere to the bottom of the flask. To get rid of the suspension cells and debris, the culture medium was transferred into a falcon and centrifuged. The cell monolayer was gently washed with PBS. After centrifugation, half of the supernatant was transferred back to the flask along with an equal amount of fresh medium. The half medium change was performed every 2 days to maintain the beneficial growth factors secreted by the cells and to ensure enough fresh medium nutrients. A gentler cell detachment solution, Accutase instead of trypsin, was used for subculturing primary GBM cells. Additionally, cell scrapers were used to detach cells from the bottom properly. The other steps were the same as described before in the cell line subculturing process.

2.2.1.3 Cryopreservation and thawing cells

GBM cell lines and primary cells were preserved at their low passages. After cell harvesting, $2x10^6$ cells suspended in 1ml freezing medium (80% culture medium with an additional 10% FCS and 10% DMSO) were transferred into a cryovial. In order to

achieve a steady rate of cooling, the vials were firstly put in a Nalgene Mr. Frosty Freezing Container and stored at -80°C for at least 24h before transferred into the liquid nitrogen tank for longer preservation.

For reviving cells, the cryovial removed from nitrogen storage was placed in a 37°C water bath for thawing. Right after it was fully defrosted, the cell suspension was slowly dripped into a 15ml tube containing 10ml pre-warmed 20% FCS culture medium. Centrifuge the tube at 1700rpm for 5 min, discard the supernatant, and rewash the cells with another 10ml of fresh medium to better remove the remaining DMSO. Culture the cells in 20% FCS medium for 2 days before changing to the regular 10% FCS culture medium.

2.2.1.4 PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donor blood. Blood was taken using Li-heparin tubes and subsequently diluted with PBS. 15ml histopaque-1077 solution was put into each 50ml falcon with filter and centrifuged at 2000rmp for 2min to get the solution down below the filter. Transfer 25-30ml diluted blood into each falcon, in which case the blood stayed on top of the filter (Figure 1A). Centrifuge at 2000rpm for 17min with a brake of 4. After centrifugation, the contents in the falcon presented as a sequence of layers, which from top to bottom were plasma-PBMCs-histopaque-filter-histopaque-erythrocytes and granulocytes (Figure 1B). Collect and discard 70-80% of the plasma fraction without disturbing the PBMCs layer. Pour the remaining supernatant above the filter into a new 50ml falcon without the filter. Fill up the falcon with PBS and centrifuge at 1500rpm for 10min. Discard the supernatant and wash with PBS for another 2 times. Resuspend the cell pellet with 1ml PBS and count the cell number using a Vi-CELL analyzer. RPMI medium supplemented with 10% FCS, 1% HEPES buffer solution, 1% L-Glutamine, and 1% penicillin/streptomycin was applied to all approaches culturing PBMCs.

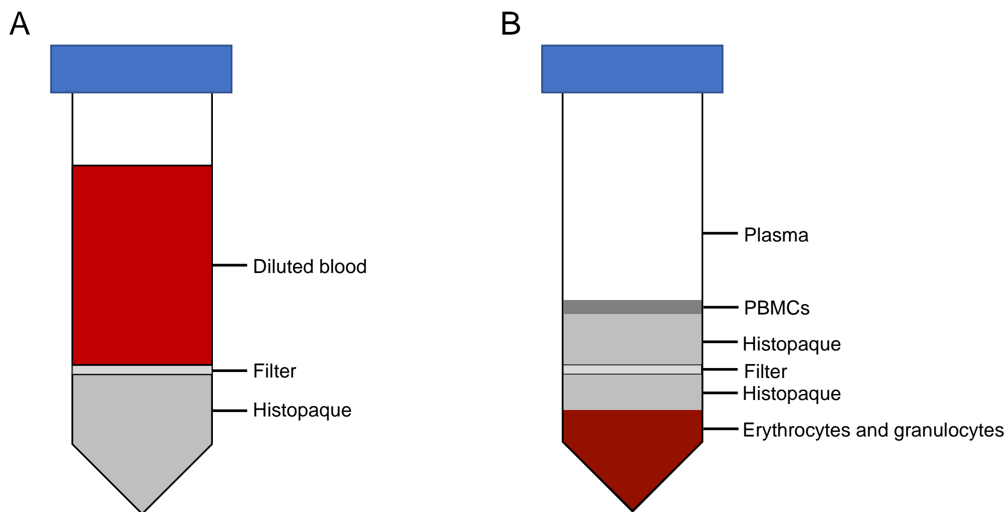

Figure 1 Schematic illustration of PBMC isolation by density gradient centrifugation. (A) Before centrifugation. (B) After centrifugation.

2.2.1.5 GBM-PBMC co-culture system

T98G cells were seeded at a density of 4×10^5 cells/well in a 6-well plate with 2ml DMEM culture medium/well. On the next day, 5×10^6 viable PBMC cells with 12.5µl Dynabeads Human T-Activator CD3/CD28 were added on top of GBM cells along with the medium change of 3ml RPMI medium per well, thus formed the direct co-culture system (Figure 2A). The final tumor-cell/PBMC ratio was about 1:10. For non-contact cell interaction, ThinCert™ cell culture inserts with 0.4µm pore size were used to separate PBMCs from GBM cells as indirect co-culture (Figure 2B). Co-cultures were treated with or without Anakinra at a concentration of 1µg/ml. GBM cells and PBMCs were also seeded separately as controls.

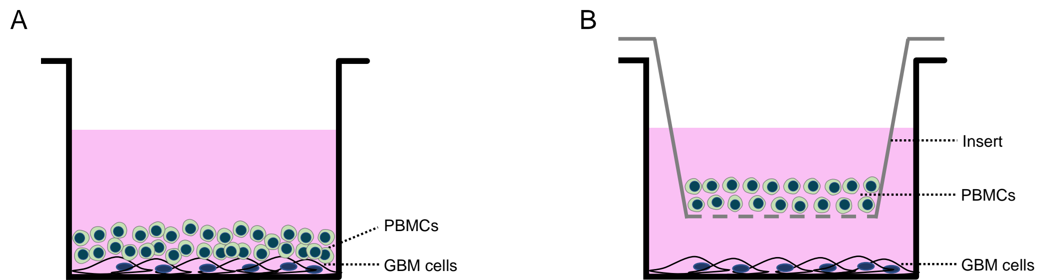

Figure 2 Schematic illustration of GBM-PBMC co-culture system. (A) Direct co-culture. (B) Indirect co-culture.

Culture plates were then put in a hypoxic condition of 5% O2 and 40mmHg CO2 at 37°C mimicking the tumor microenvironment. After 24h or 48h incubation, the culture medium was collected and preserved at -80°C for subsequent ELISA or as a conditioned medium. Cells were harvested for functional assays or lysed in RNAqueous lysis buffer or protein lysis buffer.

2.2.2 T cell isolation

PBMCs harvested from 48h indirect co-culture incubation were subsequently proceeded for T cell purification. Non-target cells were labeled with magnetic microbeads using the Pan T Cell Isolation Kit according to the manufacturer's manual magnetic labeling protocol. In brief, PBMCs were first incubated with Pan T Cell Biotin-Antibody Cocktail and then with Pan T Cell MicroBead Cocktail to label the non-T cells. Purified T cells were obtained by depletion of the labeled cells using the program "Depletes" in an AutoMACS Pro Separator. (Figure 3)

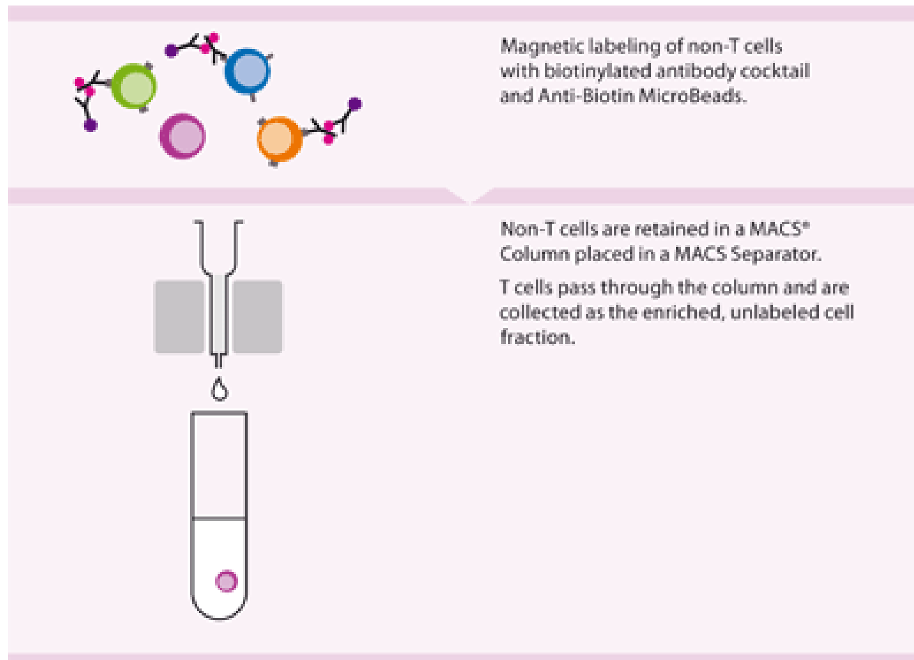

Figure 3 Schematic illustration of T cell isolation using magnetic microbeads by negative selection (picture source: Miltenyi Biotec official website).

2.2.3 Quantitative RT-PCR

2.2.3.1 RNA extraction

Invitrogen™ RNAqueous™ Total RNA Isolation Kit was used for RNA extraction. Cultured cells were harvested and lysed in 300ul lysis buffer for each sample. Cell lysates were stored at -80°C if not processed for RNA extraction immediately. The isolation procedures were following the manufacturer's protocols as follows:

- Heat an aliquot of nuclease-free water at 75°C for later use.
- Add 300μl of 64% ethanol to the cell lysate and mix it well by vortexing.
- Transfer the lysate/ethanol mixture into a filter cartridge inserted in a collection tube. Centrifuge at maximum speed for 20s to enable the mixture through the filter.
- Discard the flow-through and save the cartridge and tube for subsequent washing steps.
- Apply 700μl wash solution 1 to the filter cartridge. Centrifuge at maximum speed for 20s and discard the flow-through.
- Apply 500μl wash solution 2/3 to the filter cartridge. Centrifuge at maximum speed

for 20s and discard the flow-through.

- Repeat washing with 500µl wash solution 2/3 - centrifuge at maximum speed for 1min.
- After discarding the flow-through, briefly centrifuge the tube to remove any remaining wash solution on the filter cartridge.
- Transfer the cartridge into a fresh collection tube.
- Apply 40µl preheated 75°C nuclease-free water onto each filter.
- Centrifuge at maximum speed for 30s. The extracted RNA was in the eluate.

Subsequently, the TURBO DNA-free kit was used to remove DNA contamination in the RNA sample. For each sample, RNA was mixed with 4µl 10X TURBO DNase Buffer and 1-2µl of TURBO DNase Enzyme, and then incubated at 37°C for 30min. After incubation, add 4µl DNase Inactivation Reagent and incubate at room temperature with intermittent vortexing for 5min. Centrifuge the sample at 1000g for 1.5min. Carefully transfer 30µl supernatant to a new 0.5ml Eppendorf Safe-Lock tube without disturbing the pellet. RNA sample measurements were conducted using a NanoDrop 2000 spectrophotometer.

2.2.3.2 cDNA synthesis

A group of RNA samples were diluted with RNase-free water to the same concentration in 10µl for cDNA synthesis. Up to 1µg RNA was applied in a single reaction. For a 20ul volume reaction, 10µl RNA dilution was mixed with 1µl Oligo dT Primer, 1µl Random Hexamers, and 1ul dNTP in a 0.5ml nuclease-free microtube. Tubes were loaded on the Eppendorf Mastercycler and initiated Program cDNA65, which stands for 5min incubation at 65°C. Afterwards, the tubes were put on ice for at least 1min for cooling down. Continue to add in each tube the second reagent mixture, which consists of 4µl 5x First-Strand-Buffer, 1µl 0.1M DTT, 1µl RNAse OUT, and 1µl SuperScript Reverse Transcriptase. The incubation Program cDNAsupe was applied, which was the course of 25°C for 5min, 50°C for 45min, and 70°C for 15min. Eventually, 20µl of cDNA for

each sample was generated.

2.2.3.3 qRT-PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to quantify RNA gene expression by monitoring the amplification of the synthesized cDNA during polymerase chain reaction in real time. For each well of the 96-well PCR plate, 5μl of the cDNA sample dilution (10ng/well) was mixed with 15μl of Mastermix consisting of 4.4μl nuclease-free water, 0.2μl of each forward and reverse primer for the targeted gene, 0.2μl of the corresponding UPL probe and 10μl FastStart Essential DNA Probes Master. The 96-well plate was then placed in a LightCycler 480 to run the PCR protocol, which comprised of the initial denaturation (95°C for 10min), the 50 cycles of amplification (95°C for 10 s, 60°C for 30 s, and 72°C for 1 s) and the final cooling (40°C for 30s). The target gene expression was determined by the LightCycler® 480 Software using relative quantification analysis. TATA Box Binding Protein (TBP) and Succinate Dehydrogenase Subunit A (SDHA) were applied as housekeeping genes. Primer sequences and UPL probe numbers are provided in Table 1.

Table 1 Primer sequences and UPL probe numbers for qRT-PCR

Target	UPL probe number	Primer sequence
TBP	87	forward: 5′-GAACATCATGGATCAGAACAACA-3′
		reverse: 5′-ATAGGGATTCCGGGAGTCAT-3′
SDHA	132	forward: 5′-GAGGCAGGGTTTAATACAGCA-3′
		reverse: 5′-CCAGTTGTCCTCCTCCATGT-3′
IL-1β	41	forward: 5′-GAGGCACAAGGCACAACAG-3′
		reverse: 5′-CCATGGCTGCTTCAGACAC-3′
CCL2	40	forward: 5′-AGTCTCTGCCGCCCTTCT-3′
		reverse: 5′-GTGACTGGGGCATTGATTG-3′
COX2	2	forward: 5′-GCTTTATGCTGAAGCCCTATGA-3′
		reverse: 5′-TCCAACTCTGCAGACATTTCC-3′
BCL-2	6	forward: 5′-ACAGAGGATCATGCTGTACTTAAAAA-3′
		reverse: 5′-TTATTTCATGAGGCACGTTATTATTAG-3′
STAT3	4	forward: 5′-GGCCATCTTGAGCACTAAGC-3′
		reverse: 5′-CGGACTGGATCTGGGTCTTA-3′
IL-22	6	forward: 5′-CAACAGGCTAAGCACATGTCA-3′
		reverse: 5′-ACTGTGTCCTTCAGCTTTTGC-3′
IL-17	8	forward: 5′-TGGGAAGACCTCATTGGTGT-3′
		reverse: 5′-GGATTTCGTGGGATTGTGAT-3′
IFNγ	21	forward: 5′-GGCATTTTGAAGAATTGGAAAG-3′
		reverse: 5′-TTTGGATGCTCTGGTCATCTT-3′
IL-4	38	forward: 5′-TGCCTCACATTGTCACTGC-3′
		reverse: 5′-GCACATGCTAGCAGGAAGAAC-3′
IL-10	67	forward: 5′-TGCCTTCAGCAGAGTGAAGA-3′
		reverse: 5′-GCAACCCAGGTAACCCTTAAA-3′
GZMB	37	forward: 5′-GGGGGACCCAGAGATTAAAA-3′
		reverse: 5′-CCATTGTTTCGTCCATAGGAG-3′
PRF1	26	forward: 5′-CACTCACAGGCAGCCAACT-3′
		reverse: 5′-GGGAGTGTGTACCACATGGA-3′
IL-8		Roche RealTime Ready Single Assay ID 103136

2.2.4 Flow cytometry

All cytometric analyses, including proliferation assay and apoptosis assay, were carried out using a BD FACSCanto II flow cytometer. Data were analyzed by FlowJo software, version v10. The FACS buffer formula was 1% bovine serum albumin (BSA)

in PBS solution.

2.2.4.1 Proliferation assay

Ki-67, a nuclear protein encoded by the MKI67 gene, has widely been used as an excellent marker for cell proliferation. Ki-67 protein is detectable in all active phases of the cell cycle, including G1, S, G2, and mitosis, whereas it is absent in the quiescent status (G0 phase). In this study, Alexa Fluor 488 anti-human Ki-67 antibody was used for cell staining. The isotype control was Alexa Fluor 488 Mouse IgG1, κ antibody. The percentage of Ki-67 positive cells was quantified by flow cytometry analyses as an indicator of proliferation rate. The detailed steps of the staining protocol are as follows:

- Dilute absolute ethanol with 30% double-distilled water to produce 70% ethanol and keep in -20°C fridge for later use.
- Harvest cells from culture plates and wash twice with PBS by centrifuging at 400g for 5min. Discard the supernatant.
- Add 3ml precooled 70% ethanol onto the cell pellet drop by drop while vortexing.
- Continue to vortex for 30s and incubate the cells in ethanol at -20°C for 1h for fixation and permeabilization.
- Wash with FACS buffer for three times and resuspend the cell pellet in 200µl buffer.
- Transfer 100ul cell suspension into the FACS tube and mix with 2.5µl Ki-67 antibody or isotype antibody for staining. Incubate in the dark for 30min at room temperature.
- Wash twice with FACS buffer and resuspend in 500µl buffer for cytometric detection.

2.2.4.2 Apoptosis assay

Apoptosis, a controlled process of cell death, is characterized by nuclear chromatin condensation and fragmentation, cell shrinkage and loss of cellular membrane asymmetry. However, necrosis is a form of unregulated cell death with disrupted

plasma membrane and subsequent release of cellular components. In this study, Violet Chromatin Condensation/Dead Cell Apoptosis Kit was used for apoptotic cell detection. There were two main components in the kit. The Vybrant DyeCycle Violet stain was permeable through the cell membrane to stain on the chromatin, resulting in higher fluorescence signals in apoptotic cells than viable cells due to chromatin condensation. The SYTOX AADvanced stain binds to nucleic acid but is membrane-impermeant, which means only necrotic cells with loss of cytomembrane integrity could be labeled. With the combination of these two dyes, it is feasible to distinguish viable, apoptotic, and necrotic cell populations.

1μM working solution of Vybrant DyeCycle Violet stain was prepared by diluting with deionized water. The vial of SYTOX® AADvancedTM dead cell stain substance was dissolved in 200μl DMSO to produce a 500μM working solution. Cells were harvested from culture plates and washed twice with HBSS. For each sample, cells were resuspended in 1ml HBSS and mixed well with 1μl of the Vybrant DyeCycle Violet stain working solution and 1μl of SYTOX® AADvancedTM dead cell stain working solution. After 30min of incubation on ice, the stained cells were immediately analyzed by a flow cytometer without further washing steps.

2.2.5 Chemotaxis Assay

The chemotaxis feature of GBM cells was measured using Ibidi μ-Slides Chemotaxis according to the manufacturer's application protocol (Figure 4). The slides and all media used were pre-warmed in the incubator a day before conducting the experiment for gas equilibration to avoid air bubbles. T98G cells or primary GBM cells were harvested from different culture treatments. The cell pellet was resuspended in FCS-free culture media, and cell concentration was adjusted to 3×10^6 cells/ml. 10μl cell suspension was loaded into the central channel of the slide. All filling ports were closed with plugs, and the slide was placed in a petri dish with a wet cloth inside to maintain

humidity during overnight incubation. After cell adhesion, cells seeded in the central channel were gently washed twice with 10µl fresh medium without FCS. The adjacent two reservoirs were also filled with FCS-free medium (65µl/reservoir). Subsequently, half the volume of one side of the reservoirs was replaced by 20% FCS culture medium (30µl). Thus, a linear gradient was formed, and FCS was served as a chemoattractant. The slide was immediately mounted into the stage-top incubator to maintain 37°C temperature, while cell migration was observed using an inverted microscope through the central channel (magnification: 10×). Cell images were recorded every 10 minutes for 24 hours. Cell tracking was performed manually using ImageJ Manual Tracking Plugin. Data were analyzed using the Chemotaxis and Migration Tool. For data interpretation, the Forward Migration Index on the x-axis (FMIx) was determined as an indicator for chemotactic effect. It represents the forward migration of cells directed to the chemoattractant. The computational formula is shown in figure 4B.

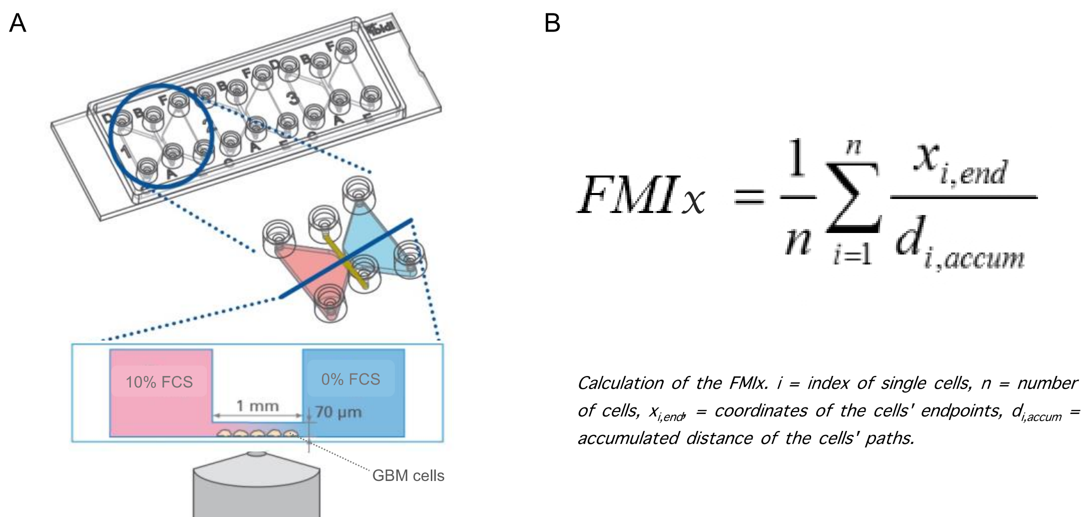

Figure 4 Chemotaxis assay using Ibidi µ-slides. (A) Schematic illustration of the chemotaxis assay. (B) Calculation of FMIx. (Picture source: Ibidi chemotaxis protocol)

2.2.6 Wound Healing Assay

T98G cells were seeded in 24-well plates (200,000 cells/well) with DMEM culture medium. When the cells had grown to full confluence, the medium was changed to

FCS-free DMEM for synchronization. On the next day, a scratch was manually made using a pipette tip in the middle of each well, forming a defined wound on the cell monolayer. The wells were gently washed with media to remove cell debris. Fresh DMEM medium containing 10% FCS was again applied in wells with different treatments: untreated as native control, +IL-1β (10ng/ml), or +IL-1β (10ng/ml) and Anakinra (1µg/ml). After 12h incubation, cell movement towards the wound was observed under an inverted microscope, and pictures were taken to compare cell migration under different treatments.

2.2.7 Transwell invasion assay

2.2.7.1 Cell preparation

T98G cells were seeded in three 25cm^2 culture flasks (500,000 cells/flask) with DMEM culture medium. After cell adhesion, two out of the three flasks were added with IL-1β (10ng/ml), of which one flask was also treated with Anakinra (1µg/ml). Hence, the three flasks of T98G were labeled as native, IL-1β, IL-1β+Anakinra, respectively. Cells were then incubated in the hypoxia chamber for 24h. On the next day, all culture medium was replaced with FCS-free DMEM medium for synchronization. After overnight incubation, the cells were ready for functional transwell assay using the CytoSelect Collagen Cell Invasion Assay Kit (Figure 5).

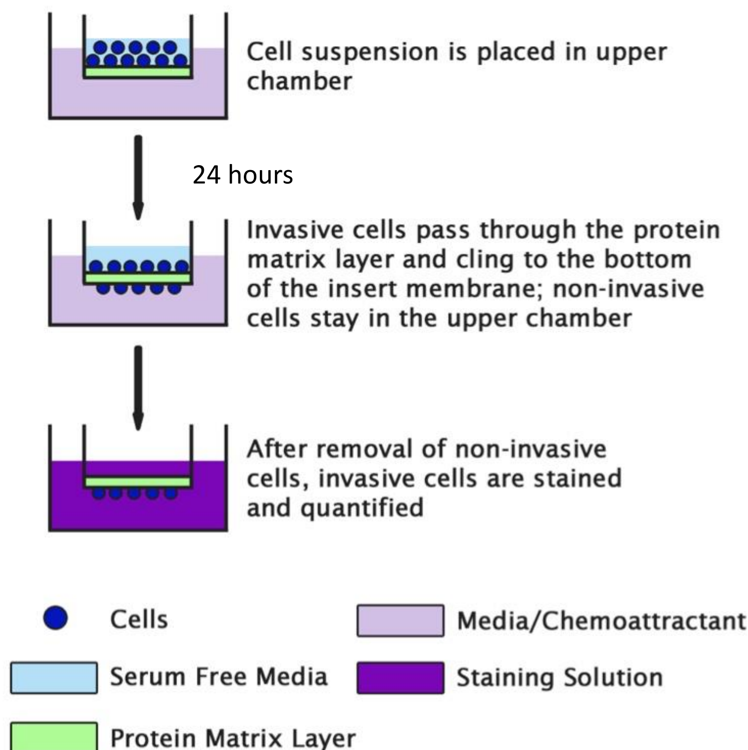

Figure 5 Schematic illustration of transwell invasion assay (picture source: CytoSelect^{TM} invasion assay protocol).

2.2.7.2 Assay Protocol

- Warm up the collagen invasion chamber plate at room temperature for 10min under sterile conditions.
- 300µl FCS-free DMEM media was added onto the cell culture inserts (pore diameter: 8µm) for collagen rehydration.
- Harvest the primed cells from flasks and adjust the cell suspension at the concentration of 400,000cells/ml in FCS-free DMEM medium.
- After 30min of rehydration at room temperature, replace 250µl rehydration media with 250µl cell suspension in each insert without disturbing the collagen layer.
- Add 500µl 10% FCS DMEM media to the lower well of the plate and incubate for 24h.
- On the next day, aspirate the media from the inserts. Gently swab the upper side of the insert membrane using wet cotton-tipped swabs to clear non-invasive cells.

- Transfer each insert to a new well containing 400µl Cell Stain Solution. Incubate for 10min.
- Gently wash the inserts in a beaker of water to remove the stain solution and wait for the inserts to air dry.
- Transfer each insert to a clean well containing 200µl Extraction Solution and incubate for 10min.
- Transfer 100µl Extraction solution from each well to a 96-well microplate, and the optical densities were measured at a wavelength of 560nm in a FilterMax F3 MultiMode Microplate Reader.

2.2.8 ELISA

Cytokine levels of IFNγ, IL-17, IL-22, and IL-10 in the culture medium were determined by Enzyme-Linked Immunosorbent Assay (ELISA). Supernatants from indirect co-cultures were harvested and subsequently preserved at -80°C. Frozen supernatants were slowly thawed on ice and centrifuged to remove debris before conducting the assay. 100µl of each sample was applied in the 96 microwell plate provided by ELISA kits for quantification. Respectively, LEGEND MAX™ Human ELISA Kit was used for IFNγ and IL-17 detection, and ELISA MAX™ Deluxe Set was used for IL-22 and IL-10 detection. The principle of the test is a sandwich ELISA based on specific antigen-antibody binding, which generates an antibody-antigen-antibody “sandwich”. The assay procedure followed the manufacturer's protocol. Optical density was measured at the absorbance of 450nm by a FilterMax F3 MultiMode Microplate Reader. All samples were run in duplicates, and mean values were taken for analysis.

2.2.9 Protein analysis

2.2.9.1 Protein extraction

For lysis buffer preparation, the 10X Cell Lysis Buffer was diluted with RNase-free

water to obtain a 1X solution supplemented with 1% protease inhibitor and 1% phosphatase inhibitor. T98G cells were harvested from 12-well plates and dissolved in 100μl of prepared protein lysis buffer for each sample. The lysates were incubated on the ice for 5min and briefly sonicated in an ultrasonic water bath for 1min. Incubate on ice again for 5min and centrifuge in a precooled microfuge (15,000rpm, 10min, 4°C). The supernatants that contained protein were transferred into new Eppendorf-tubes and stored at -80°C or immediately continued with subsequent quantification.

2.2.9.2 BCA assay

Protein quantification was measured using the Pierce BCA Protein Assay Kit. The assay principle is the colorimetric detection of Cu^{+1}, which is produced in the reaction of protein with Cu^{+2} in an alkaline environment, using a reagent containing bicinchoninic acid (BCA). Before conducting the assay, the Albumin Standard of 2mg/ml was diluted with BCA Reagent A in different ratio to prepare a set of albumin standards: 2mg/ml, 1mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml and 0mg/ml. BCA working reagent was prepared by mixing BCA reagent A with BCA reagent B at the ratio of 50:1. The transparent flat-bottom 96-well microplate was used for the assay. 10μl of protein sample or standard solution and 200μl BCA working reagent was applied in each well. Shake the microplate briefly for 30s on a plate shaker for mixing and then incubate at 37°C for 30min. Absorbance at 550nm was measured by a FilterMax F3 MultiMode Microplate Reader. A four-parameter logistic curve for standards was generated to determine sample concentrations. All samples and standards were measured in duplicates.

2.2.9.3 SDS-PAGE and Western Blotting

The formula of the gels and buffers in need before the assay is listed in table 2. 15μg of each protein sample was diluted with 6x SDS loading buffer and nuclease-free water. The samples were heated at 95°C for 5min for denaturation and then kept on ice for

later use. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins by their molecular masses. The method was conducted by preparing a polyacrylamide gel consisting of a 12% separating gel part and a 4% stacking gel part. The denatured protein samples were loaded equally into the lanes of the stacking gel, as well as 5μl PAGERuler Protein Ladder to provide visible markers on the gel and membrane. The gel was placed in a BIO-RAD Electrophoresis Chamber. The 1X SDS running buffer was filled in the chamber until it fully covered the gel. A voltage of 100V was applied to the gel for protein electrophoresis, which took about 2h.

Next, the separated proteins were transferred onto a PVDF membrane using a BIO-RAD Trans-Blot Turbo System. The membrane was then incubated in TBS-Tween-20 (TBST) with 5% non-fat milk for 1h at room temperature to block nonspecific binding. Primary antibodies for BCL-2 (Clone: D55G8) or β-Actin (Clone: 13E5) were diluted in TBST containing 1% non-fat milk, in which the membrane was incubated overnight in a 4°C fridge. The next day, the membrane was washed in TBST (3 times, 10min/wash) before incubated with the secondary HRP-linked antibody for 2h at room temperature. Wash again with TBST 3x10min to remove excess antibodies. Immunoblotting was visualized using the Clarity^{TM} Western ECL Substrate kit by digital imaging. Densitometric quantification was analyzed using ImageJ.

Table 2 Gel and buffer preparation for SDS-PAGE

Separating gel (12%)	dd H_2O	5.963ml
	Separating gel buffer	4.5ml
	30% Acrylamide	7.2ml
	10% SDS	180μl
	10% APS	180μl
	TEMED	18μl
Stacking gel (4%)	dd H_2O	4.8ml
	Stacking gel buffer	2ml
	30% Acrylamide	1064μl
	10% SDS	80μl
	10% APS	80μl
	TEMED	8μl
Separating gel buffer	1.5M Tris-HCl pH 8.8 in 100ml dd H_2O	
Stacking gel buffer	0.5M Tris-HCl pH 6.8 in 100ml dd H_2O	
10X SDS running buffer	Tris	30.3g
	Glycine	144g
	SDS	10g
	dd H_2O	1L
10X TBS	Tris	60.5g
	NaCl	87.6g
	HCl	till pH 7.5
	dd H_2O	1L
TBST	10X TBS	100ml
	dd H_2O	900ml
	Tween-20	1ml
6X SDS loading buffer	Tris-HCl	5.91g
	SDS	6g
	100% Glycerol	48ml
	2-Mercaptoethanol	9ml
	Bromophenol blue	30mg
	dd H_2O	fill to 100ml

2.2.10 Statistical analysis

All data were analyzed using GraphPad Prism 7 software and presented as mean ± SEM unless stated otherwise. The Student's *t*-test or Wilcoxon signed-rank test were chosen for intergroup comparison, depending on whether the datasets coincided with normal distribution. p-value < 0.05 was considered as statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant).

3. Results

3.1 IL-1β upregulates the pro-inflammatory gene expression in GBM

Firstly, we aimed to investigate the impact of IL-1β-induced inflammation on GBM in vitro. To this end, a dose-finding analysis was conducted to evaluate the inflammatory response of GBM cells upon different doses of IL-1β stimulation. T98G glioblastoma cells were treated with 10, 20, or 40 ng/ml of IL-1β. Since hypoxia is one of the prominent hallmarks of the GBM microenvironment that exacerbates the tumor-related inflammation and contributes to tumor progression[182, 183], we chose a moderate hypoxic condition to perform all experiments in this study. The culture plates were incubated in moderate hypoxia of 5% O_2 for 24h until the cells were harvested and lysed for subsequent RNA isolation. The mRNA gene expression of pro-inflammatory cytokines IL-6 and IL-8 was measured by qPCR. As a potent inflammatory mediator, IL-1β significantly upregulated IL-6 and IL-8 mRNA levels in T98G, even at the lowest 10ng/ml concentration (Figure 6: IL-6: +25.63-fold±1.0, p=0.0015; IL-8: +257.11-fold±20.89, p=0.0065). The upregulation was not concentration-dependent, as 20 or 40ng/ml IL-1β led to similar results (Figure 6). Hence, IL-1β 10ng/ml concentration was chosen for the subsequent experiment setup, since a higher dose would not result in a stronger inflammatory response.

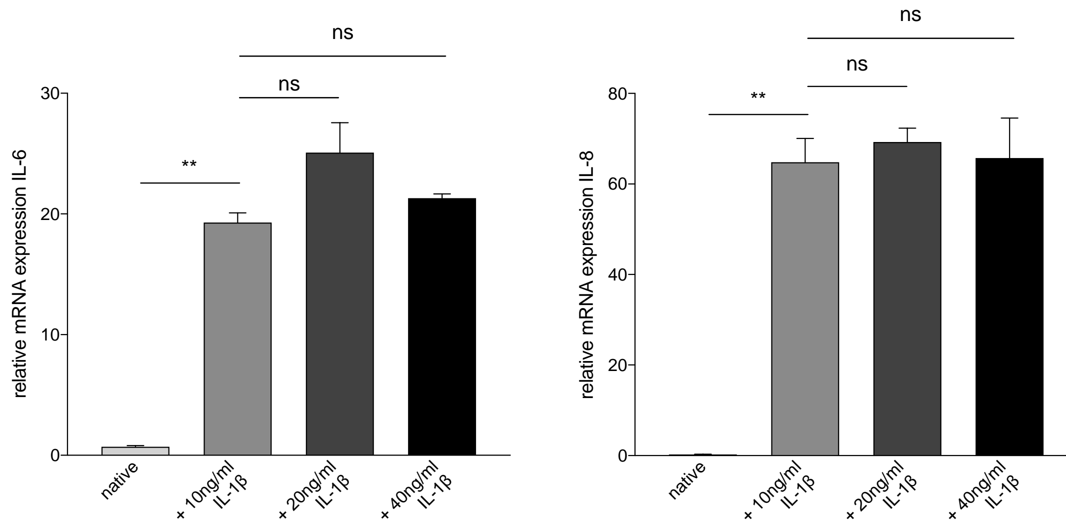

Figure 6 IL-1β stimulates pro-inflammatory cytokine gene expression in T98G.
T98G cells were treated with different concentrations of IL-1β (10, 20 or 40 ng/ml). Cells were lysed for RNA isolation after 24h hypoxic incubation. Pro-inflammatory gene expression was analyzed by qRT-PCR (n=3). ***p*<0.01.

3.2 Anakinra inhibits the IL-1β-induced upregulation of pro-inflammatory gene expression in both GBM and PBMC

Next, we investigated if the recombinant IL-1 antagonist anakinra was able to suppress this IL-1β-induced inflammation in GBM. Therefore, we treated T98G cells with 10ng/ml IL-1β and different concentrations of anakinra (Ana, 10ng/ml, 100ng/ml, or 1µg/ml) for 24h. Anakinra was administered 3h after IL-1β stimulation, since inflammation always occurs ahead of any anti-inflammatory treatments in vivo. Numerous inflammatory cytokines were reported to be associated with tumor aggressiveness, of which we chose a few prominent ones to measure their gene expression - IL-1β, COX2, CCL2, and IL-8. The mRNA expression of these pro-inflammatory genes was markedly upregulated upon IL-1β stimulation (Figure 7: IL-1β: +97.18-fold±23.08, *p*=0.0244; COX-2: +14.83-fold±6.12, *p*=0.0940; CCL2: +3.84-fold±0.32, *p*=0.0013; IL-8: +522.0-fold±146.25, *p*=0.0234). After anakinra administration, the expression levels of those pro-inflammatory targets declined to different extents. With the highest concentration of anakinra, the inflammatory markers

were drastically reduced to the basic level (Figure 7: IL-1β: −98.9%±23.5%, p=0.0245; COX-2: −92.4%±37.7%, p=0.0919; CCL2: −82.9%±8.8%, p=0.0025; IL-8: −99.8%±28.0%, p=0.0234). Anakinra inhibits the IL-1β-induced inflammation in a dose-dependent manner. Thus, anakinra in a concentration of 1µg/ml was chosen for further experiments.

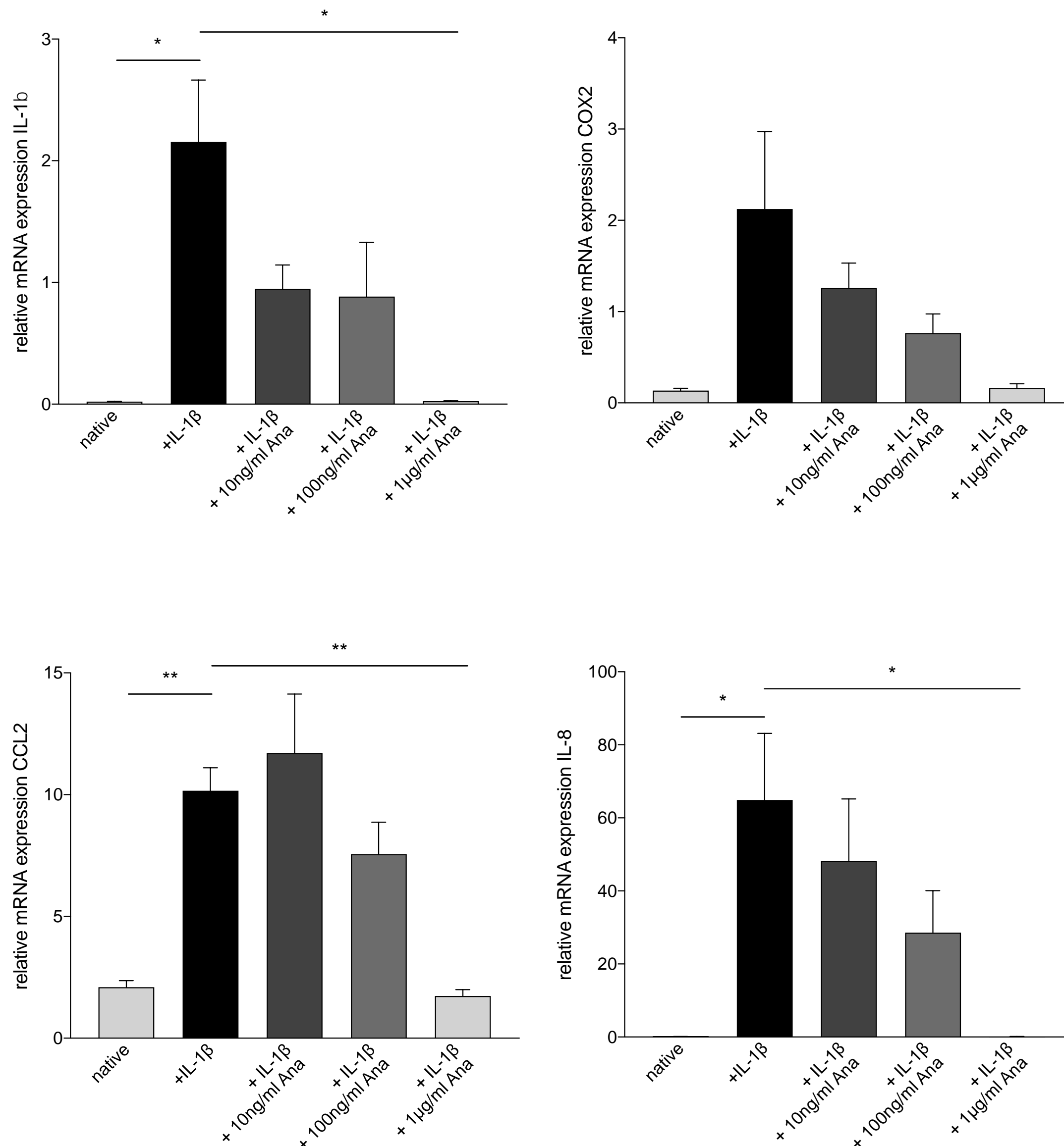

Figure 7 Anakinra inhibits the IL-1β-induced pro-inflammatory cytokine gene expression in T98G in a dose-dependent manner.

T98G cells were stimulated with 10ng/ml IL-1β. After 3h, different concentrations of anakinra (Ana, 10ng/ml, 100ng/ml or 1µg/ml) were applied. Cells were lysed for RNA isolation after 24h hypoxic incubation. Pro-inflammatory gene expression was analyzed by qRT-PCR (n=4). *p<0.05, **p<0.01.

Then we assessed the impact of anakinra on both primary GBM cells and PBMCs in response to IL-1β-stimulated inflammation. Primary GBM cells were treated with IL-1β (10ng/ml), with or without Anakinra (1μg/ml) administration 3h later. After 24h hypoxic incubation, the GBM cells were harvested for RNA isolation, and mRNA expression of target genes was quantified. The heterogeneous primary cells exhibited a similar gene expression change as the T98G cell line. IL-1β stimulation drastically elevated the pro-inflammatory gene expression of IL-1β, COX2, CCL2 and IL-8 in primary GBM cells (Figure 8: IL-1β: +13.73-fold±3.62, p=0.0312; COX-2: +17.2-fold±5.65, p=0.0381; CCL2: +2.90-fold±1.65, p=0.0312; IL-8: +11.1-fold±3.64, p=0.0312). However, the upregulation was significantly diminished by the administration of anakinra (Figure 8: IL-1β: −80.6%±25.2%, p=0.0312; COX-2: −92.3%±30.9%, p=0.0405; CCL2: −70.3%±40.2%, p=0.0312; IL-8: −85.7%±29.9%, p=0.035).

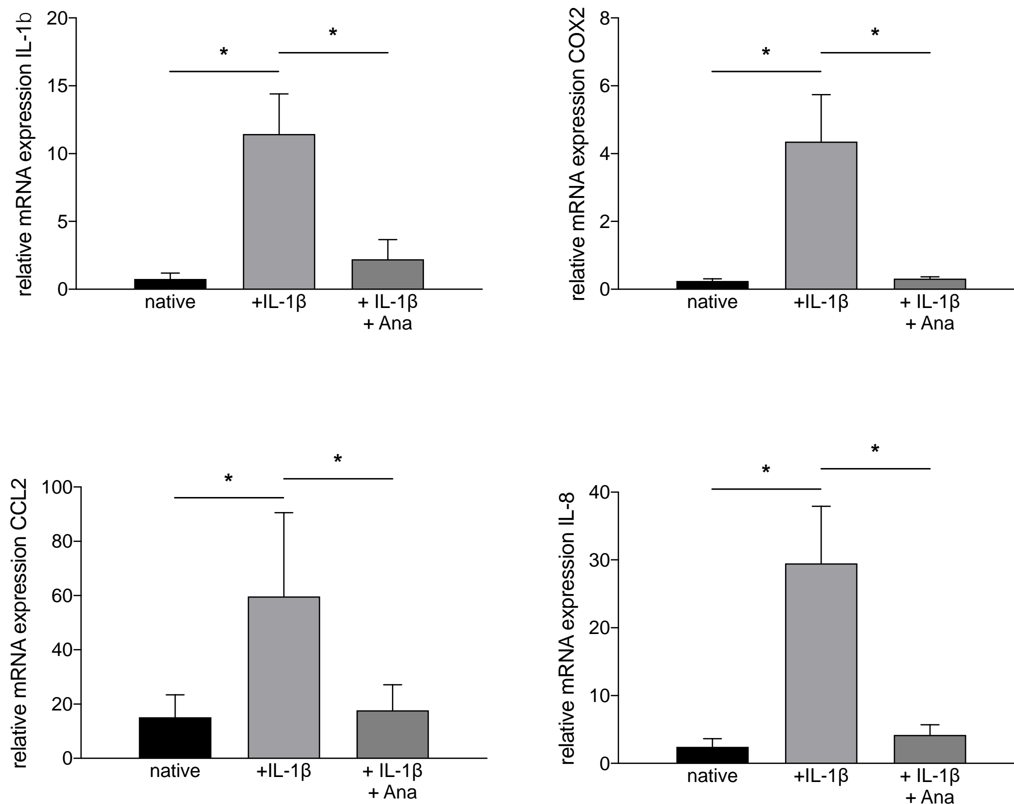

Figure 8 Anakinra suppresses the IL-1β-induced pro-inflammatory cytokine gene expression in primary glioblastoma cells.

Primary GBM cells were stimulated with IL-1β (10ng/ml), and with or without the administration of anakinra (Ana, 1μg/ml) 3h later. Then cells were incubated in hypoxic conditions for 24h. The mRNA gene expression was analyzed by qRT-PCR (n=6). *p<0.05

PBMCs are composed of monocytes and lymphocytes, which are the major players of both anti-tumor immunity and tumor-associated inflammation[184]. Thus, we isolated PBMC cells from healthy human donor blood and studied the effect of anakinra on these immune cells. PBMCs were treated with IL-1β (10ng/ml) alone or with both IL-1β and anakinra (1μg/ml, 3h later) for 24h. All pro-inflammatory cytokine gene expressions detected were markedly boosted by IL-1β stimulation (Figure 9: IL-1β: +15.1-fold±1.31, p=0.0003; COX-2: +2.57-fold±0.27, p<0.0007; CCL2: +8.77-fold±1.63, p=0.0057; IL-8: +8.22-fold±1.22, p=0.0026), while anakinra was able to significantly suppress the IL-1β-induced upregulation (Figure 9: IL-1β: −93.3±8.1%, p=0.0003; COX-2: −69.0%±7.9%, p=0.0009; CCL2: −88.8%±15.9%, p=0.005; IL-8: −86.0%±11.8%, p=0.0078).

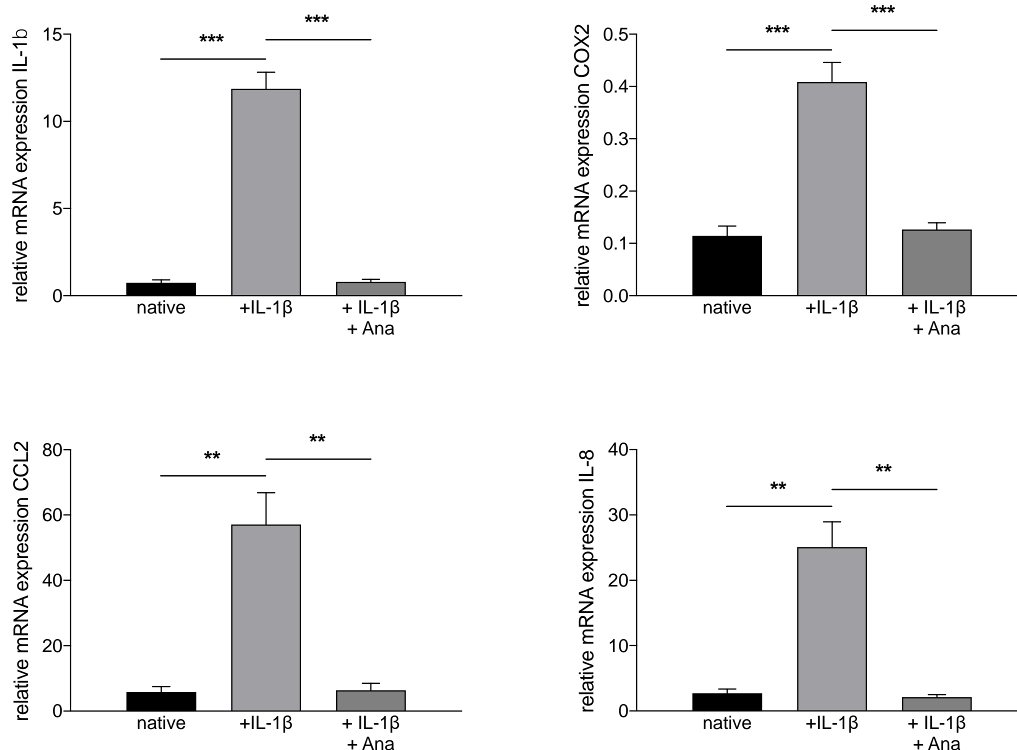

Figure 9 Anakinra reduces the IL-1β-induced pro-inflammatory cytokine gene expression in PBMCs.

PBMCs were stimulated with IL-1β (10ng/ml), and with or without the administration of anakinra (Ana, 1μg/ml) 3h later. Then cells were incubated for 24h. The mRNA gene expression was analyzed by qRT-PCR (n=5). **p<0.01, ***p<0.001.

The transcription factor - STAT3 exerts a leading role in regulating the tumor downstream signaling in response to inflammation[67]. STAT3 activation also contributes to tumor growth and metastasis[66, 185]. Therefore, we quantified the STAT3 expression in IL-1β-stimulated T98G. As shown in Figure 10, IL-1β upregulated STAT3 only on a small scale (Figure 10: +19.7%±9.3%, p=0.1246), and this effect was marginally reduced by anakinra (Figure 10: -3.9%±5.7%, p=0.5419). Although the trends were in line with our expectations, the differences did not reach statistical significance.

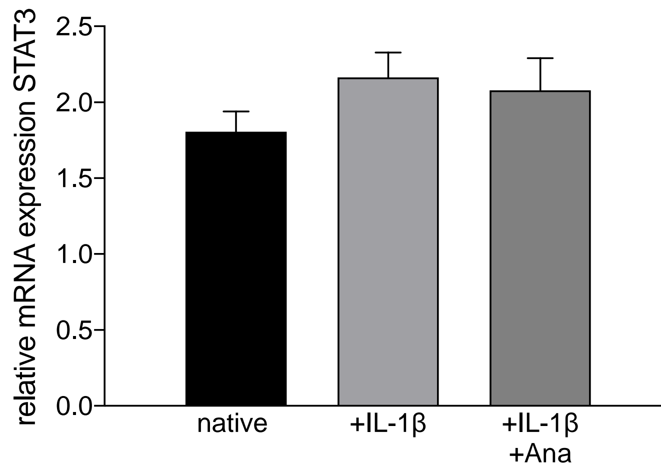

Figure 10 STAT3 gene expression in T98G upon IL-1β stimulation with or without anakinra. T98G cells were stimulated with IL-1β (10ng/ml), and with or without the administration of anakinra (Ana, 1μg/ml) 3h later. Then cells were incubated in hypoxic conditions for 24h. The mRNA gene expression was analyzed by qRT-PCR (n=4). p=ns.

3.3 Anakinra suppresses IL-1β-promoted GBM proliferation, migration and invasion.

Since we had validated the pro-inflammatory role of IL-1β in upregulating pro-inflammatory gene expressions in GBM cells and characterized the anti-inflammatory function of anakinra in abrogating the IL-1β-stimulated inflammation, we moved on to the next question – whether this change of inflammatory status would influence tumor malignancy. To this end, functional assays regarding proliferation, migration and invasion were conducted.

3.3.1 Anakinra inhibits IL-1β-promoted tumor proliferation in GBM

T98G cells were treated with IL-1β in the presence or absence of anakinra. After 48h incubation under hypoxic conditions, cells were harvested and stained intracellularly with the Ki-67 antibody or the corresponding isotype control. Ki-67 expression was measured by flow cytometry. Since Ki-67 protein is only detectable in all the active phases of the cell cycle, Ki-67 positive cells were considered as proliferating cells[186]. Upon IL-1β stimulation, T98G cells presented a higher Ki-67 expression compared to the untreated native control (Figure 11B: +19.8%±17.4%, p=0.3375). This IL-1β-induced upregulation of Ki-67 expression was significantly reduced by anakinra (Figure 11B: −21.0%±5.1%, p=0.0265), which indicates an inhibited tumor proliferation rate with anakinra treatment.

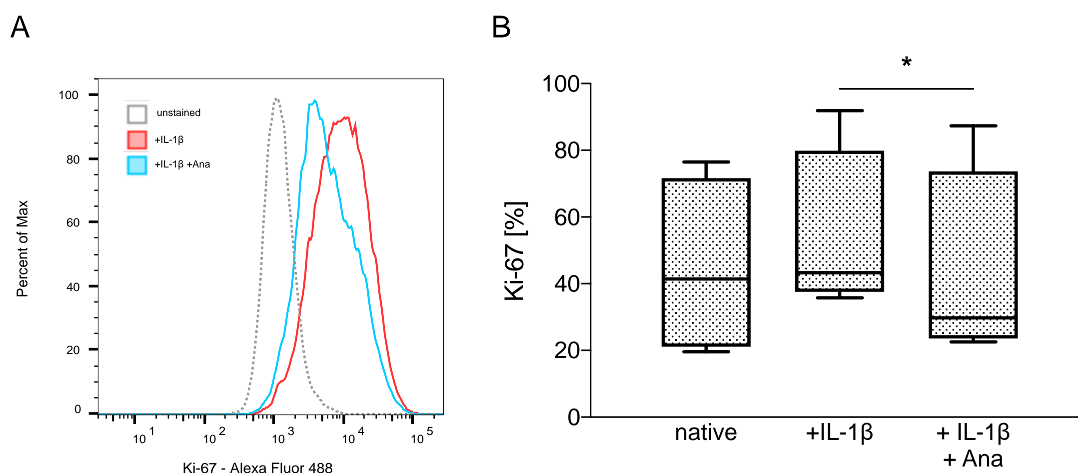

Figure 11 Anakinra reduces the IL-1β-upregulated Ki-67 expression in T98G.
T98G cells were treated with IL-1β (10ng/ml) or with both IL-1β and anakinra (Ana, 1μg/ml, 3h later) for 48h in hypoxic conditions. Ki-67 expression level was assessed by flow cytometry. (A) Representative FACS histogram showing Ki-67 fluorescence intensity. (B) Box plot comparing the percentage of Ki-67 positive cells of T98G (n=4). *p<0.05.

3.3.2 Anakinra mitigates IL-1β-promoted tumor migration and invasion in GBM

Cancer cell migration and invasion are important hallmarks of GBM malignancy. There are multiple experimental methods to evaluate tumor metastasis in vitro, of which we chose the 2D wound healing assay, transwell invasion assay, and the directed chemotaxis assay to investigate GBM migration and invasion.

3.3.2.1 Wound healing migration assay

A wound healing assay, also called a scratch assay, is often used to assess the tumor cell migration on the 2D level. Confluent T98G cells were starved in FCS-deprived culture medium overnight for synchronization before the cell monolayer was scratched by a pipette tip to generate a cell-free gap. Then the starving medium was replaced by normal culture medium with or without IL-1β or anakinra. This way, cells migrated from the sides towards the wound area in differently treated media. Images of the wound area were recorded at 0h and 12h after scratching. The wound gaps at 0h were approximately at the same width. After 12h, more cells had migrated into the gap under IL-1β stimulation. Treatment with anakinra was able to reduce the pro-migratory effects induced by IL-1β (Figure 12). This result indicates that IL-1β stimulation promotes GBM migration, and that anakinra is able to counteract this inflammation driven tumor aggressiveness.

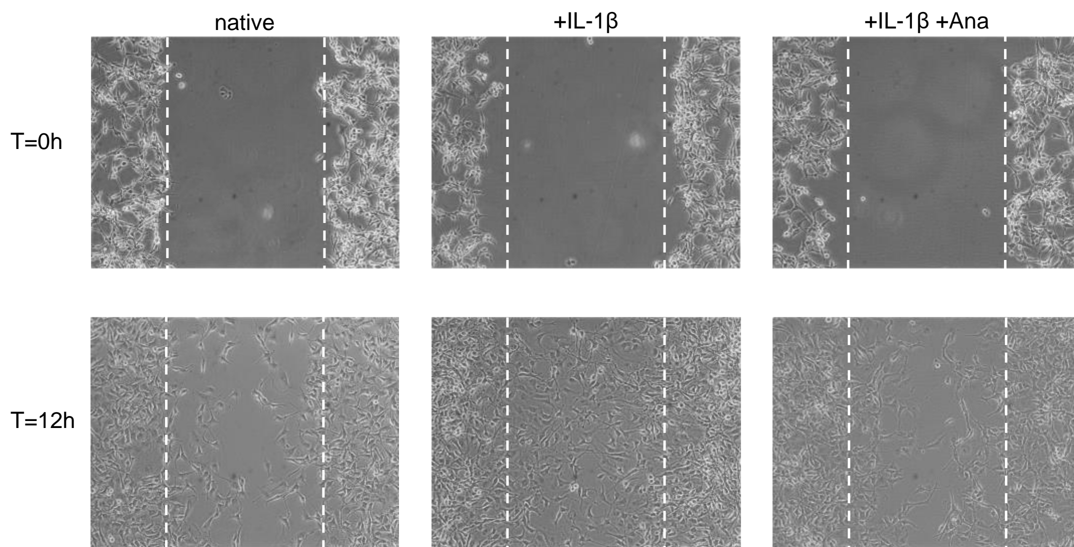

Figure 12 Wound healing assay: anakinra mitigates the IL-1β-promoted cell migration in T98G. Cells were migrating under native condition, or with IL-1β/ IL-1β+anakinra (Ana) treatment during 12h hypoxic incubation. Representative images at two time-points of the wound closing process (beginning: 0h, end: 12h) are shown. The white dotted lines indicate the initial cell-free wound (n=6).

3.3.2.2 Chemotaxis assay

Since a wound healing assay only allows to examine the undirected cell migration, we next performed a chemotaxis assay to explore the directed tumor cell migration towards a chemoattractant, using both T98G cells and primary glioblastoma cells. GBM cells were seeded without treatment or pre-treated with IL-1β or with both IL-1β and anakinra for 24h under hypoxic conditions. Then the primed cells were harvested and seeded into the three chambers of Ibidi chemotaxis slide. The chemoattractant (FCS) was added into one side of the reservoir forming a stable linear gradient. Cell movement was observed by acquiring images every 10min for 24h using time-lapse microscopy. In the cell trajectory plots, IL-1β pre-treated T98G cells showed more active and directed movements compared to the native control and IL-1β+anakinra pre-treated cells (Figure 13A). Statistically, IL-1β treatment led to an increased FMIx value compared to the native control (Figure 13B: +1.49-fold±0.98, p=0.2289), and a drastic reduction of FMIx was found in IL-1β+anakinra treatment (Figure 13B: -1.13-

fold±0.37, *p*=0.048). In summary, IL-1β stimulation induced a promoted chemotactic migration of tumor cells, and anakinra substantially suppressed this effect.

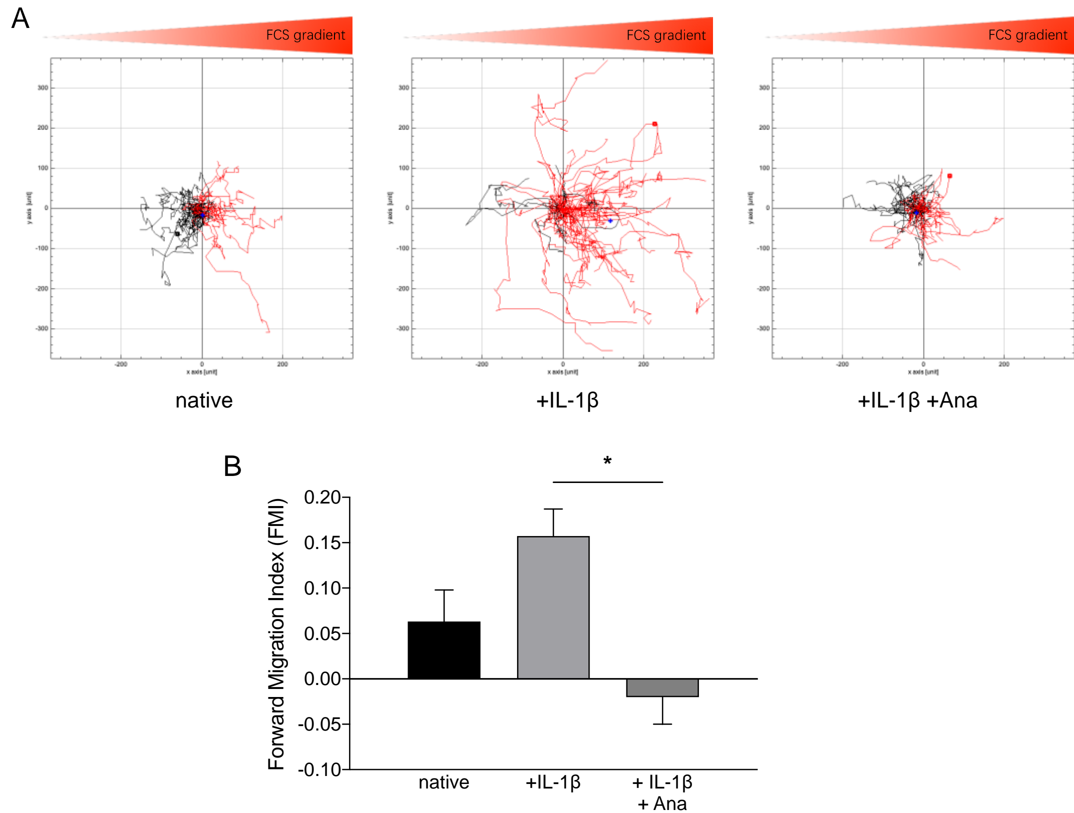

Figure 13 Chemotaxis assay: anakinra mitigates the IL-1β-promoted chemotactic migration in T98G.

T98G cells were treated with IL-1β (10ng/ml) or with both IL-1β and anakinra (Ana, 1μg/ml) for 24h in hypoxia before harvested and reseeded for chemotaxis. (A) An exemplary trajectory plots of chemotactic cell movements. At least 40 cells were manually tracked in each plot. (B) The forward migration index on the x-axis (FMIx) was calculated as cell displacement towards the chemoattractant (n=4). *$p<0.05$.

Next, we conducted chemotaxis assays using primary GBM cells. In line with our findings of the GBM cell line, primary cells exhibited increased directed migratory capability with IL-1β treatment as well as inhibited migration by anakinra, however, without reaching statistical significance. (Figure 14: +IL-1β vs. native: +2.67-fold±1.40, p=0.1914; +IL-1β+Ana vs. +IL-1β: -0.54-fold±0.31, p=0.2262).

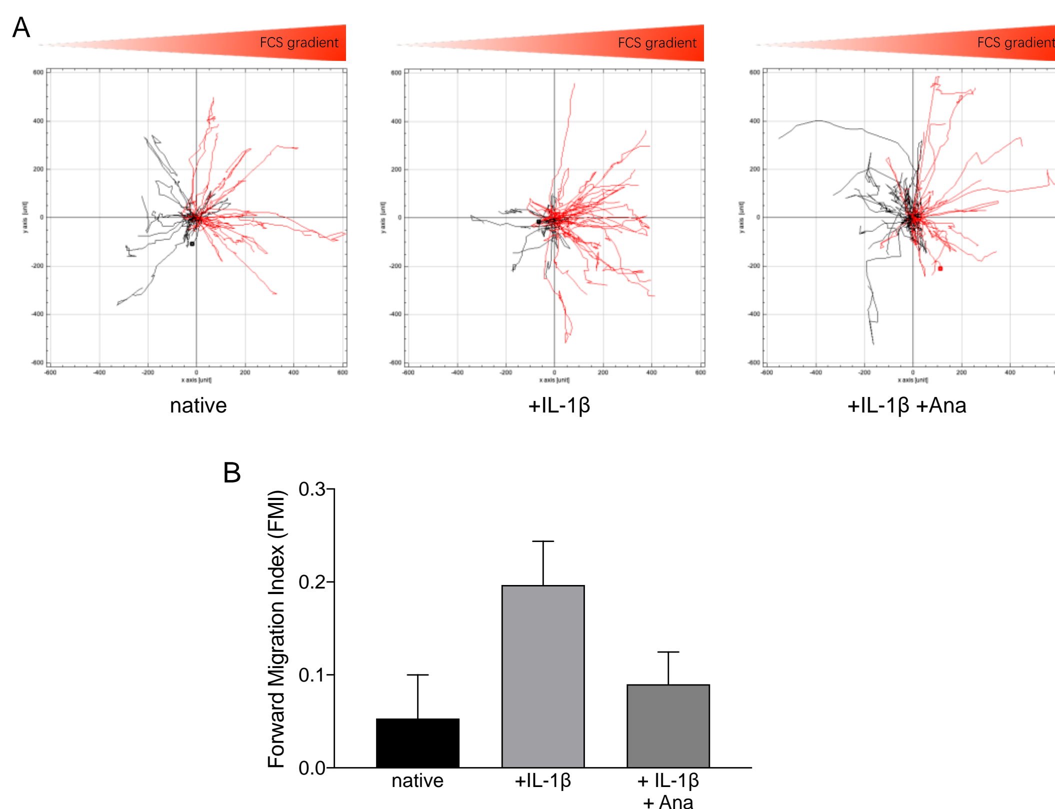

Figure 14 Chemotaxis assay of primary glioblastoma cells upon IL-1β stimulation with or without anakinra.

Primary GBM cells were treated with IL-1β (10ng/ml) or with both IL-1β and anakinra (Ana, 1µg/ml) for 24h in hypoxia before harvested and reseeded for chemotaxis. (A) An exemplary trajectory plots of chemotactic cell movements. At least 40 cells were manually tracked in each plot. (B) The forward migration index on the x-axis (FMIx) was calculated as cell displacement towards the chemoattractant (n=3). p=ns.

3.3.2.3 Transwell invasion assay

Invasion is one of the hallmarks of tumor malignancy. Therefore, we conducted a transwell assay to evaluate not only the chemotaxis effect of the tumor cells, but also their invasive ability to migrate through a physical barrier[187]. T98G cells were seeded without any treatment or were primed with IL-1β or IL-1β+anakinra for 24h under hypoxic conditions. The pre-treated cells were then harvested and seeded into the

transwell inserts. Subsequent 24h incubation allowed the tumor cells to migrate through the insert membrane towards the chemoattractant FCS. Eventually, only the transmigrated cells on the lower side of inserts were stained, and the optical density at 560nm was measured as quantification. Hence, a higher optical density represents more invasive tumor cells. As indicated in figure 10, IL-1β significantly enhanced the invasive capacity of T89G cells (Figure 15: +37.8%±2.9%, p=0.0489). Anakinra attenuated tumor invasion as the OD value declined to the basic level upon anakinra administration along with IL-1β (Figure 15: −23.4%±2.7%, p=0.048).

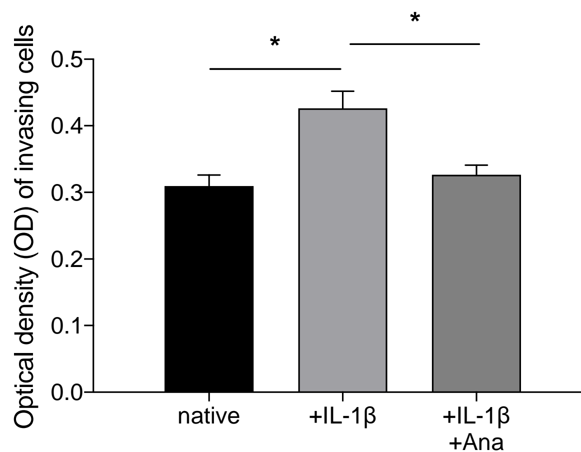

Figure 15 Transwell invasion assay showing inhibited T98G migration and invasion by anakinra.

T98G cells were pretreated with IL-1β (10ng/ml) or with both IL-1β and anakinra (Ana, 1μg/ml) for 24h in hypoxic conditions before applied to the transwell assay. Optical density at 560nm was measured as the quantification for invasive tumor cells (n=3). *p<0.05.

Taken together, IL-1β stimulation induces a more aggressive GBM phenotype with promoted proliferation and migration. More importantly, anakinra not only dampens the IL-1β-induced inflammatory response, but also ameliorates GBM malignancy.

3.4 Anakinra in GBM-PBMC co-culture

Within the tumor microenvironment, GBM tumor cells are embedded with various other cells, including immune cells. The infiltrating immune cells not only exert their anti-tumor immunity, but their crosstalk with tumor cells profoundly contributes to the tumor-

promoting inflammation[2]. Thus, we established a co-cultivation model of T98G cells and PBMC cells, in order to mimic the tumor microenvironment in vivo. For indirect co-culture, an insert with 0.4μm pores was used to separate PBMCs from GBM for respective harvesting and analyses yet allowing the interaction of cell-secreted cytokines and mediators.

3.4.1 Anakinra dampens inflammatory signaling in co-cultured GBM

T98G cells were indirectly co-cultured with PBMCs under hypoxic conditions. After 24h incubation, T98G cells were harvested for RNA isolation and pro-inflammatory gene expression measurement. Surprisingly, even without any additional stimuli, the indirect co-culture of GBM cells and PBMCs was able to upregulate the pro-inflammatory gene expressions in T98G (Figure 16: IL-1β: +70.7%±32.4%, p=0.0938; COX-2: +79.9%±51.8%, p=0.1835; CCL2: +5.81-fold±2.04, p=0.0374; IL-8: +14.11-fold±4.66, p=0.0292). Moreover, anakinra administration in the co-culture dampened this inflammatory response – all detected gene expressions were significantly decreased (Figure 16: IL-1β: −64.0% ± 19.1%, p=0.0155; COX-2: −56.3% ± 21.4%, p=0.0156; CCL2: −87.1% ± 46.0%, p=0.0235; IL-8: −91.7% ± 27.0%, p=0.0145).

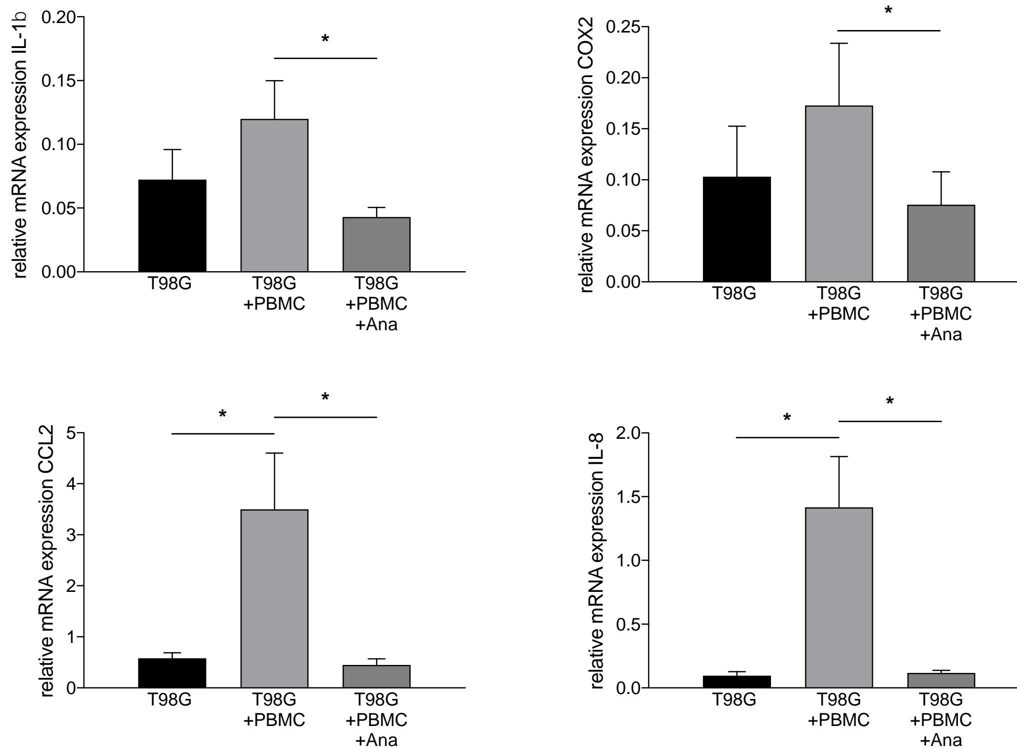

Figure 16 Anakinra suppresses the co-culture-upregulated pro-inflammatory cytokine gene expression in T98G.

T98G cells were co-cultured with PBMCs, and with or without the administration of anakinra (Ana, 1μg/ml). Then cells were incubated in hypoxic conditions for 24h. The mRNA gene expression was analyzed by qRT-PCR (n=7). *$p<0.05$.

Having found a trend in STAT3 expression induced by IL-1β stimulation and attenuated by anakinra, we further assessed whether the crosstalk of GBM and immune cells would have an impact on STAT3 activation. Confirming and even strengthening our findings with GBM cells only, the co-cultured T98G expressed a significantly higher level of STAT3 compared to the native control (Figure 17: +26.9%±5.1%, p=0.0062), while anakinra significantly reduced this STAT3 upregulation in GBM tumor cells (Figure 17: −11.0%±3.2%, p=0.0145).

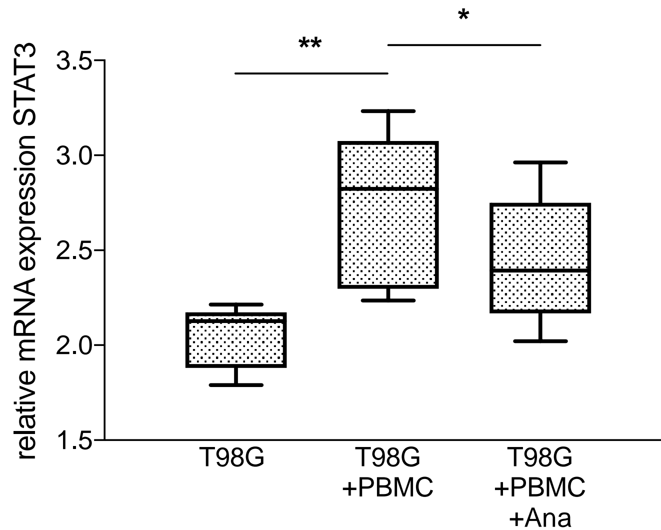

Figure 17 Anakinra reduces the co-culture-upregulated STAT3 expression in T98G.
T98G cells were co-cultured with PBMCs, and with or without the administration of anakinra (Ana, 1μg/ml). Then cells were incubated in hypoxic conditions for 24h. The mRNA gene expression was analyzed by qRT-PCR(n=7). *p<0.05, **p<0.01.

3.4.2 Anakinra suppresses T cell inflammatory signaling in co-culture

Among the infiltrating immune cells within tumor microenvironment, T lymphocytes, as the effector of adaptive immunity, play a vital role in anti-tumor immune responses[188]. On the other hand, T cell cytokine secretion has an intricate influence on tumor growth and development. Particularly, pro-inflammatory cytokines, such as IFNγ, IL-17, and IL-22, are known to induce tumor-associated inflammation that accelerates GBM progression[189, 190]. We assumed that anakinra might also regulate the T cell cytokine signaling in GBM microenvironment. Thus, T cells were extracted from the co-cultured PBMCs, and the co-culture supernatant was preserved for ELISA analysis. Inflammatory cytokine expressions were evaluated on mRNA level of the T cells as well as on protein level secreted in the respective culture medium. Pro-inflammatory cytokines IFNγ, IL-17, and IL-22 significantly decreased with anakinra treatment on both mRNA level (Figure 18A: IFNγ: −85.0%±45.4%, p=0.002; IL-17: −88.1%±21.7%, p=0.0028; IL-22: −63.2%±22.0%, p=0.002) and secreted protein level(Figure 18A: IFNγ: −13.04%±3.3%, p=0.0039; IL-17: −51.8%±20.8%, p=0.0039; IL-22: −37.1%±8.5%, p=0.0047). Conversely, the expression of anti-inflammatory cytokine

IL-10 was elevated by anakinra in co-culture treatment(Figure 18B: mRNA: +64.5%±30.0%, *p*=0.0383; protein: +15.5%±5.1%, *p*=0.0023).

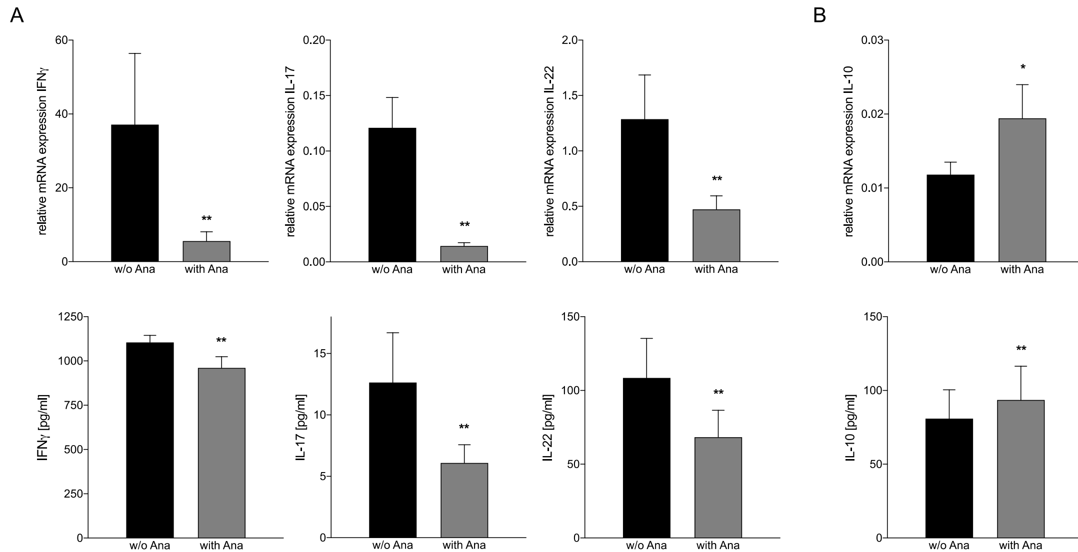

Figure 18 Anakinra decreases pro-inflammatory cytokine levels and increases anti-inflammatory cytokine level in T cells or in culture medium with co-culture treatment.
T cells were extracted from PBMCs after indirectly coculturing with T98G for 48h in hypoxic conditions, with or without anakinra (Ana, 1µg/ml). The mRNA gene expression was analyzed by qRT-PCR. Secreted cytokine level in the co-culture supernatant was quantified by ELISA. (A) mRNA expressions and protein levels of pro-inflammatory cytokines IFNγ, IL-17, and IL-22 (mRNA: n=13; protein: n=10). (B) mRNA expression and protein level of anti-inflammatory cytokine IL-10(n=10). **p*<0.05, ***p*<0.01.

Additionally, we analyzed if anakinra might influence the T cell mediated cytotoxicity. Perforin 1 and granzyme B are the essential cytotoxic effector molecules[191]. Both of their mRNA expressions in co-cultured T cells were barely affected by anakinra (Figure 19: PFR1: w/o Ana: 2.94±0.6, with Ana: 2.81±0.58; GZMB: w/o Ana: 16.19±5.78, with Ana: 13.58±3.99), suggesting that anakinra dampens the pro-inflammatory signaling in T cells without impairing its cytotoxic antitumor immunity.

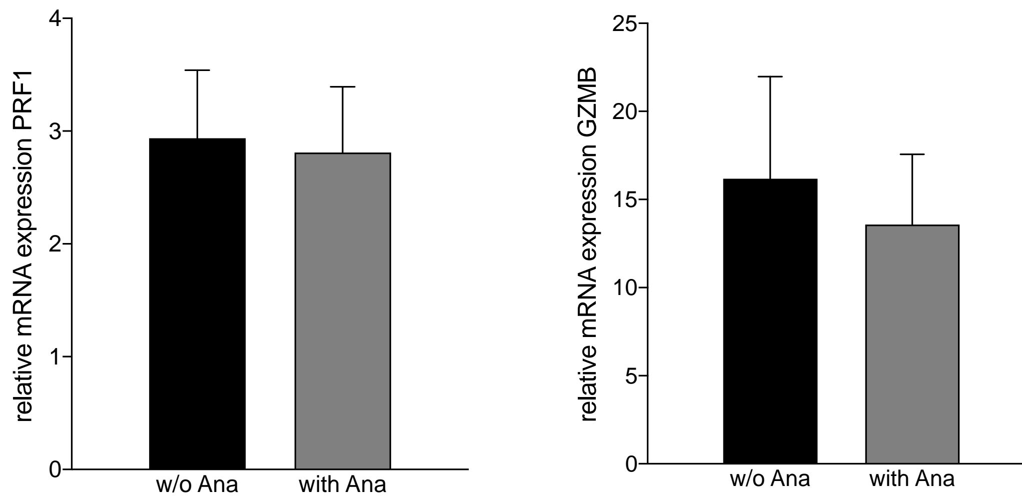

Figure 19 PRF1 and GZMB mRNA gene expressions in T cells from co-culture treatment remain unaffected by anakinra.
T cells were extracted from PBMCs after indirectly co-culturing with T98G for 48h in hypoxic conditions, with or without anakinra (Ana, 1µg/ml). The mRNA gene expression was analyzed by qRT-PCR (PFR1: n=9; GZMB: n=4). *p*=ns.

3.4.3 Anakinra inhibits tumor proliferation, migration in indirect co-culture

Anakinra presented a similar anti-inflammatory effect in the co-culture model as in IL-1β-stimulated GBM. Since we had already found out that anakinra could attenuate GBM aggressiveness by reducing the IL-1β-induced inflammation, we hypothesized anakinra might also lead to a less proliferative and migratory phenotype of GBM in co-culture.

3.4.3.1 Anakinra attenuates co-culture-enhanced tumor proliferation in GBM

T98G cells were in indirect co-culture with PBMCs for 48h in hypoxic incubation. Afterwards, the tumor cells were harvested and stained with Ki-67 antibody to assess the tumor proliferation. A higher percentage of proliferating cells was found in co-cultured T98G compared to the native (Figure 20: +52.0%±17.5%, p=0.048). Additional anakinra administration in co-culture significantly reduced the Ki-67 expression (Figure

20: −25.2%±6.8%, *p*=0.00192), suggesting the co-culture-promoted tumor proliferation was ameliorated by anakinra.

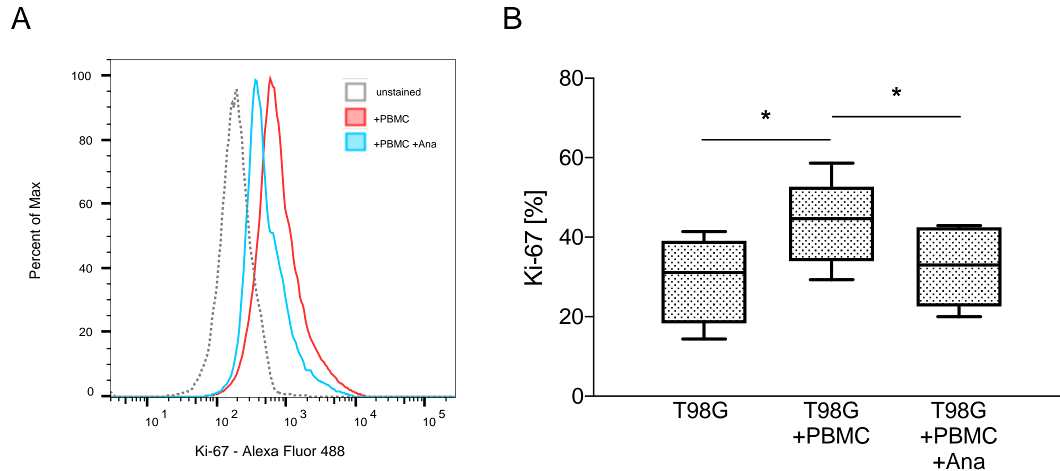

Figure 20 Anakinra reduces the co-culture-upregulated Ki-67 expression in T98G.
T98G cells were co-cultured with PBMCs, and with or without the administration of anakinra (Ana, 1μg/ml) for 48h in hypoxic conditions. Ki-67 expression level was assessed by flow cytometry. (A) Representative FACS histogram showing Ki-67 fluorescence intensity. (B) Quantification of Ki-67 positive fraction in box plot (n=5). **p*<0.05.

3.4.3.2 Anakinra mitigates co-culture-enhanced tumor migration in GBM

We next conducted chemotaxis assays to demonstrate whether the GBM-PBMC crosstalk has an impact on directed tumor migration. T98G cells were indirectly co-cultured with PBMCs for 24h in hypoxic incubation before harvested and applied to a chemotaxis assay. FCS served as the chemoattractant. Cell movement was observed for another 24h. Eventually, the cell trajectory plots exhibited a more active and directed chemotactic migration in co-cultured T98G cells compared to untreated native cells (Figure 21A). When anakinra was applied along with the co-culture treatment, the enhanced migratory movement was considerably inhibited (Figure 21A). The calculation of FMIx represents as the quantification of cell chemotactic migration (Figure 21B: +PBMC vs. native: +3.36-fold±1.77, *p*=0.1331; +PBMC +Ana vs. +PBMC: −1.09-fold±0.17, *p*=0.0081).

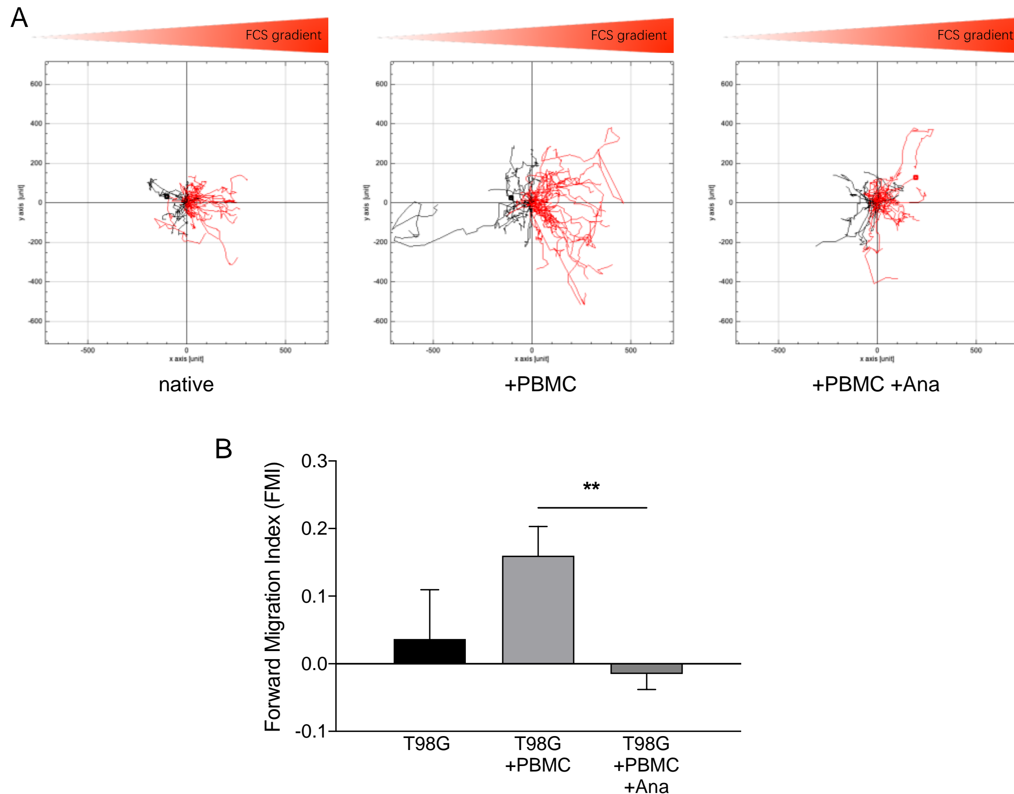

Figure 21 Chemotaxis assay: anakinra mitigates the co-culture-promoted chemotactic migration in T98G.

T98G cells were co-cultured with PBMCs, and with or without the administration of anakinra (Ana, 1μg/ml) for 24h in hypoxic conditions before harvested and seeded for chemotaxis. (A) An exemplary trajectory plots of chemotactic cell movements. At least 40 cells were manually tracked in each plot. (B) The forward migration index on the x-axis (FMIx) was calculated as cell displacement towards the chemoattractant (n=4). ***p*<0.01.

3.4.4 Anakinra increases tumor apoptosis of GBM in direct co-culture

We found that STAT3 expression in GBM was induced by co-culture and inhibited by anakinra, which led us to explore this signaling pathway further. STAT3 signaling is known to regulate cancer apoptosis via mediating the expression of Bcl-2 family proteins[71]. The disrupted balance of anti-apoptotic proteins and pro-apoptotic proteins in the Bcl-2 family contributes to dysregulated apoptosis in cancer[192]. Thus, we aimed to examine the major two members of the family: the anti-apoptotic Bcl-2 and the pro-apoptotic Bax.

Firstly, we assessed the Bcl-2 gene expression of indirectly co-cultured T98G cells.

The anti-apoptotic marker did not show any significant changes with co-culture treatment or with both co-culture and anakinra (Figure 22: T98G: 0.0067±0.0019; T98G+PBMC: 0.0061±0.0012; T98G+PBMC+Ana: 0.0058±0.0011). That made us consider that the indirect crosstalk of GBM and PBMCs without direct cell-cell contact might not be sufficient to induce apoptotic signaling. Thus, we conducted direct co-culture treatment to enable direct GBM-PBMC cell interaction. However, the conventional setup of direct co-incubation of GBM and PBMCs would raise the question of how to separate tumor cells from the co-culture for the subsequent analyses. To tackle this problem, we performed the following procedures to ensure both GBM-PBMC direct contact signaling and separated cell analysis.

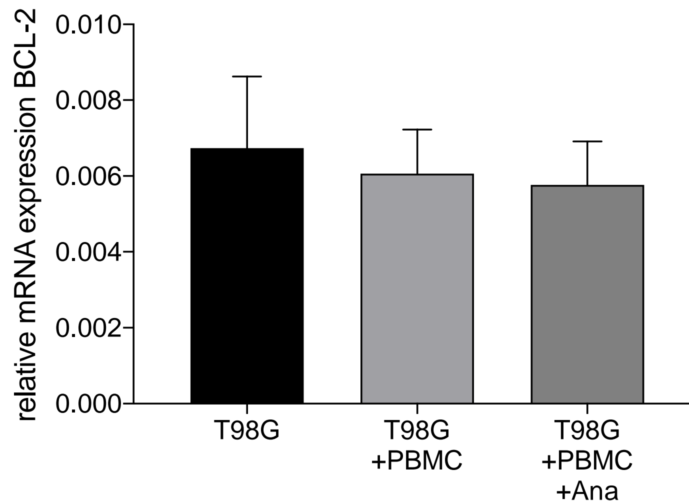

Figure 22 Bcl-2 gene expression in T98G with indirect co-culture treatment, with or without anakinra.

T98G cells were co-cultured with PBMCs, and with or without the administration of anakinra (Ana, 1μg/ml). Then cells were incubated in hypoxic conditions for 24h. The mRNA gene expression was analyzed by qRT-PCR (n=3). *p*=ns.

T98G and PBMCs were directly cultured together in RPMI medium, with or without anakinra administration. After 48h hypoxic incubation, the supernatant was harvested as the conditioned medium, which contained all the cell-secreted cytokines and mediators due to GBM-PBMC direct interaction. Subsequently, different conditioned media were respectively subjected to freshly seeded T98G cells. After another 24h incubation, the cells were harvested for mRNA, protein analysis, or apoptosis assay.

For mRNA expression analysis, the anti-apoptotic Bcl-2 and the pro-apoptotic Bax were evaluated, and Bax/Bcl-2 ratio was calculated, as it is an essential determinant of apoptosis balance in cancer[193, 194]. T98G cells incubated in co-culture conditioned medium resulted in an increased mRNA (Figure 23A: +76.9%±7.7%, p=0.0312) and protein (Figure 23B: +20.3%±14.2%) expression of Bcl-2 and a lower Bax/Bcl-2 ratio (Figure 23C: −47.6%±9.1%, p=0.0151) compared to the control without co-culture treatment. When anakinra was applied in the co-culture, T98G exhibited a downregulation of Bcl-2 (Figure 23A: mRNA: −18.3%±7.5%, p=0.0128; Figure 23B: protein: −22.5%±3.5%) and an elevated Bax/Bcl-2 ratio (Figure 23C: +36.5%±13.1%, p=0.0491).

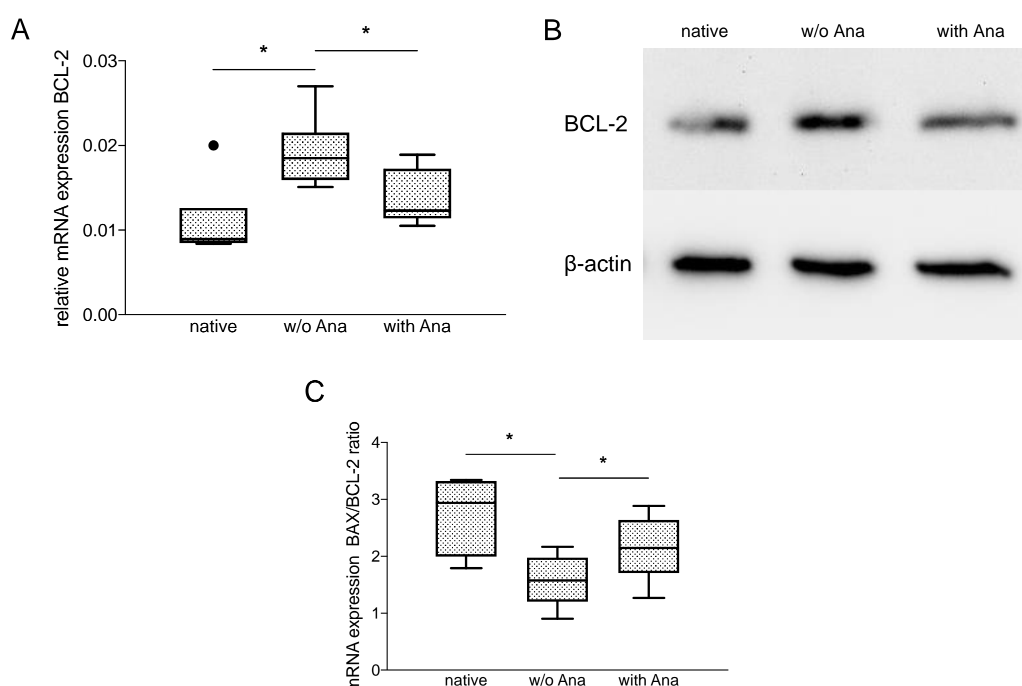

Figure 23 Anakinra decreases the anti-apoptotic marker expression in T98G with direct co-culture treatment.

T98G cells were incubated in conditioned media collected from direct co-culture of T98G and PBMCs, with or without anakinra (Ana, 1µg/ml). After 24h incubation in hypoxic conditions, all cells were harvested. The mRNA gene expression was analyzed by qRT-PCR. Protein analysis was assessed by western blot. (A) mRNA expression of Bcl-2 in T98G (n=6). (B) One representative example for protein analysis of Bcl2 and the reference protein β-actin in T98G (n=3). (C) mRNA expression of Bax/Bcl-2 ratio in T98G (n=5). *p<0.05.

Apoptosis analysis by flow cytometry was performed to further identify if the change of apoptotic-related markers would actually affect the tumor apoptotic rate. In line with the Bax/Bcl-2 expression, direct co-culture medium led to a declined percentage of apoptotic T98G cells (Figure 24: −63.7%±23.0%, p=0.0396). When anakinra interfered with GBM-PBMC crosstalk, the apoptotic rate was significantly increased (Figure 24: +43.6%±8.4%, p=0.0068).

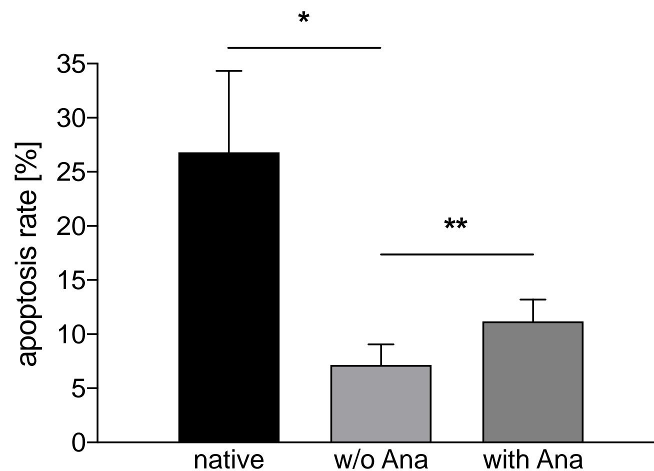

Figure 24 Anakinra induces a higher apoptosis rate in T98G with direct co-culture treatment. T98G cells were incubated in conditioned media collected from direct co-culture of T98G and PBMCs, with or without anakinra (Ana, 1µg/ml). After 24h hypoxic incubation, the percentage of apoptotic T98G cells was assessed by flow cytometry (n=6). *p<0.05, **p<0.01.

Taken together, the GBM-PBMC crosstalk induced a pro-inflammatory tumor microenvironment in a similar manner as IL-1β stimulation, which exerted a pro-tumorigenic influence on GBM cells with promoted proliferation and migration, and reduced apoptosis. Anakinra administration in addition to co-culture or to IL-1β stimulation always had inhibitory effects – down-regulation of pro-inflammatory cytokine gene expression along with attenuated tumor aggressiveness was observed.

4. Discussion

Glioblastoma is one of the most malignant types of cancer with a very poor prognosis. Despite all kinds of treatment options, the median survival time of GBM patients is only 12-15 months after diagnosis[34, 195]. Over the decades of research, tumor-associated inflammation has been revealed as an essential promotor for tumor progression in many cancers[196, 197], including glioblastoma[198]. Numerous inflammatory cytokines and mediators are produced by both tumor cells and the infiltrating immune cells, creating a pro-inflammatory tumor microenvironment[199]. The unregulated inflammation within the TME should not be mistaken for a tumor specific immune response. It is essential for the host defense against tumor that immune cells execute anti-tumor immunity from immunosurveillance to tumor clearance[200]. However, certain types of immune cells engage in dynamic crosstalk with tumor cells, resulting in a chronic inflammatory milieu that suppresses effective anti-tumor immunity and facilitates every stage of tumor development[42, 201]. The intricate network among inflammatory cells, inflammatory cytokines and the tumor plays a significant role in cancer progression.

IL-1 signaling has been identified as an important key-mediator in regulating immunity and inflammation, including orchestrating immune responses, maintaining immune homeostasis, and also causing pathological inflammatory disorders[202]. The role of IL-1 in tumor development is regarded as a double-edged sword. Within the tumor microenvironment, IL-1β can exert anti-tumor functions by activating innate and adaptive immunity. On the other hand, IL-1β-mediated chronic inflammation promotes tumor growth[203]. Both tumor cells and immune cells produce IL-1β in the autocrine and paracrine manner, contributing to immune suppression and tumor progression[203]. IL-1β was found to be overexpressed in GBM cell lines and tumor specimens. IL-1β activates the inflammatory signaling and induces the production of multiple cytokines in the tumor microenvironment[63], suggesting a link between IL-

1β-mediated inflammation and GBM progression. IL-1β-treated GBM cells have exhibited enhanced proliferation, invasion and migration[204]. Therefore, targeting IL-1 signaling in the GBM microenvironment might be a promising therapeutic approach to pursue.

In this study, we first validated the pro-tumorigenic effects of IL-1β-induced inflammation in GBM and investigated whether the recombinant IL-1 receptor antagonist anakinra could reverse the IL-1β-promoted GBM aggressiveness by targeting IL-1 signaling. Further, we established an in vitro model of the tumor microenvironment consisting of GBM tumor cells and human immune cells. Our findings unraveled that anakinra ameliorates GBM tumor malignancy by impeding the vicious cycle of self-aggravating inflammation caused by GBM-immune cell interplay.

4.1 Anakinra attenuates IL-1β-induced tumor-associated inflammation and GBM aggressiveness

IL-1β is considered a potent mediator that amplifies the inflammatory response. To validate the pro-inflammatory effects of IL-1β in the GBM microenvironment, we identified the pro-inflammatory gene expression in GBM cells and PBMCs upon IL-1β stimulation.

For the experimental setup, we chose a moderate hypoxic condition of 5% O_2 to conduct all GBM-cell-related cultivation. Hypoxia is a prominent pathophysiological feature within the GBM tumor microenvironment due to the enhanced metabolic demands and an inadequate oxygen supply. Although a more severe hypoxic condition of O_2 concentration, even less than 1%, was detected in the tumor site[205, 206], we intended to maintain the cell viability of both GBM cells and immune cells in vitro that enables active cellular interactions. An extremely deficient oxygen level in the central zone of the tumor is always accompanied by massive necrotic tumor cells. Besides, a

concept of “Goldiloxygen zone” has been proposed, that an oxygen range of 5-8% O_2 is optimal for in vitro cell culture research, where the oxygen level is just right for viable metabolism with less oxidative damage compared to normoxia[207].

Our findings have shown that IL-1β boosted the mRNA expression of the tumor-promoting inflammatory cytokines IL-1β, IL-8, CCL2 and COX2, in both GBM cell line and primary tumor cells. Moreover, IL-1β can also lead to a similar upregulation of cytokine expressions in healthy donor PBMC cells. These results indicate that IL-1β can not only activate GBM tumor cell inflammatory signaling, but also stimulate the immune cells to produce abundant pro-inflammatory mediators. Of note, by blocking the IL-1 receptor, anakinra administration sufficiently suppressed the IL-1β-induced inflammatory gene upregulations in GBM and PBMCs. Since IL-1β is known to induce IL-6 production and STAT3 activation[55, 208], which further engage in accelerating GBM progression and aggressiveness[209], the expression level of STAT3 in GBM cells was also quantified. We found a tendency of STAT3 upregulation with IL-1β stimulation, and a slight reduction with additional anakinra application, however, without reaching statistical significance.

Mounting evidence has revealed that the pro-inflammatory cytokines in the tumor microenvironment are associated with a more aggressive GBM phenotype[210]. IL-1 signaling has emerged as a driver of cancer progression[211]. Thus, we suspected that IL-1β might induce an inflammatory GBM milieu that promotes GBM aggressiveness. Therefore, anakinra might be a promising therapeutic candidate by targeting IL-1β-dependent signaling.

Functional assays were conducted to assess GBM malignancy in vitro. Ki-67 expression analyzed by flow cytometry was applied as a proliferation marker. Tumor migration was first evaluated by a wound healing assay, which provides a convenient, inexpensive and widely used approach for cell migration analysis. However, this

method only measures random, undirected cell migration. To study the directed migration of tumor cells towards a chemical stimulus, we performed a more sophisticated chemotaxis system that enables real-time recording of chemotactic cell motility using time-lapse microscopy. Additionally, the transwell assay allowed us to quantify the migrated tumor cells towards a chemoattractant across a physical barrier, indicating both of its directional movement and invasive ability.

Indeed, the results of functional assays supported our hypothesis that IL-1β induced a more aggressive GBM phenotype with increased tumor proliferation, migration and invasion. On the other hand, anakinra was able to reverse the IL-1β-promoted GBM malignancy - less proliferating cells and inhibited migratory and invasive movements were found. Our results were in line with one previous study that implicated a relation with IL-1β and GBM malignancy - IL-1β has shown pro-tumorigenic effects with enhanced proliferation, migration and invasion in GBM cell lines, and these promoting effects were inhibited by IL-1Ra[167].

A list of previous studies could provide possible molecular mechanisms and signaling pathways to support our findings. IL-1β exerts its pro-oncogenic functions by activating the NF-κB and MAPK pathways and stimulating extensive secretion of pro-inflammatory cytokines, including IL-1β itself and other mediators as IL-8, CCL2 and COX2. The transcription factor NF-κB signaling in GBM involves multiple biological processes, including maintenance of GBM stem-like cells, promotion of tumor invasion and therapy resistance[212]. Aberrant activation of MAPK signaling pathway in GBM patients is associated with increased tumor proliferation and shorter survival time[213]. IL-8 binds to its receptors CXCR1/2 on GBM cells and facilitates tumor proliferation and invasion[214]. Within the TME, a dialog between GBM and microglia has been found. GBM-produced CCL2 activates the CCR2 signaling on microglia to induce excessive IL-6 production, which in turn acts on GBM cells and enhances tumor invasiveness[86]. COX2 and its major downstream product PGE2 contribute to GBM

progression regarding tumor proliferation, invasion, apoptosis and immunosuppression[215].

4.2 Anakinra ameliorates GBM aggressiveness by dampening the inflammatory GBM-immune cell crosstalk

The cancer-related inflammation is generated by the crosstalk between tumor cells and infiltrating immune cells. Therefore, we next aimed to investigate the effects of anakinra in a newly established in vitro model mimicking the tumor microenvironment by indirectly co-cultivating GBM cells with immune cells under moderate hypoxic conditions. Interestingly, even without any extraneous stimuli, the indirect interaction with PBMC cells could elevate the mRNA expression of pro-inflammatory markers IL-1β, IL-8, CCL2 and COX2, and the transcription factor STAT3 in GBM. The cytokine communication between GBM and immune cells, even without any direct cell contact, led to a similar inflammatory response as IL-1β stimulation in GBM cells. More importantly, anakinra was able to impede this GBM-PBMC inflammatory crosstalk by mitigating the pro-inflammatory gene expression.

Among different types of immune cells, T cells in the GBM microenvironment are the major effectors of adaptive anti-tumor immunity. However, malignant tumors, like GBM, can induce T cell dysfunction, resulting in their inadequate anti-tumor immunity and even tumor-facilitating properties[216]. A number of T cell-secreted cytokines are associated with T cell activities and GBM tumor progression in regard to inflammation-induced tumorigenesis. For instance, it has been revealed that the pro-inflammatory IL-17 and IL-22 possess promoting roles in tumorigenesis and cancer metastasis via activating the oncogenic transcription factor STAT3[141, 142, 217]. Thus, we investigated whether anakinra can influence the cytokine production in the co-cultured T cells, and found out that anakinra suppressed the pro-inflammatory cytokines IFNγ, IL-17, and IL-22 on both mRNA expression and secreted protein level. Besides, the

anti-inflammatory cytokine IL-10 was increased by anakinra treatment. Meanwhile, the expression level of the key effector molecules for T cell cytotoxicity, PFR1 and GZMB, remained unaffected by anakinra. These results have indicated that blocking the IL-1 pathway restrains the pro-inflammatory T cell phenotype in the GBM tumor environment by regulating its cytokine production without compromising the anti-tumor cytotoxicity.

Based on the above results, anakinra has led to a less inflamed phenotype of both GBM cells and T cells in the indirect co-culture setting. The question whether this modulation of inflammatory cytokine expression profile would influence GBM aggressiveness was evaluated with functional assays regarding tumor proliferation, migration and apoptosis. Indirect GBM-PBMC co-culture resulted in increased proliferation rates and enhanced chemotactic migration in GBM cells. IL-22 induces tumor proliferation and reduces cell apoptosis in GBM by activating the transcription factor STAT3[142], which further upregulates its downstream anti-apoptotic protein Bcl-2[71]. We examined the anti-apoptotic marker Bcl-2 in GBM cells. However, it was not affected by indirect co-culture. Therefore, we established a direct co-culture model and found out that direct co-culture treatment resulted in increased Bcl-2 expression, as well as a reduced apoptotic rate in GBM cells. The direct cell-cell contact with immune cells may be required to induce apoptotic change of GBM cells. Thus, the IL-22/STAT3/Bcl-2 signaling pathway that regulates tumor apoptosis has been validated.

Of note, anakinra reversed the effects of the indirect/direct co-culture treatment and induced a less aggressive GBM phenotype with inhibited proliferative and migratory competence and increased apoptotic rate.

The present work has underscored the link between GBM aggressiveness and the inflammation generated by tumor-immune cell crosstalk. Our findings of multiple inflammatory cytokine expressions and their association with GBM malignancy are

mostly concordant with previous studies. IFNγ, as the hallmark cytokine of Th1 cells, has demonstrated its anti-tumor effects in immune surveillance and tumor clearance[218]. However, other evidence has revealed a “dark side” of IFNγ in promoting tumor immunoevasion and progression[126]. In GBM, IFNγ induces the immune checkpoint PD-L1 expression on GBM cells and contributes to tumor immune escape[127]. The paradoxical functions of some cytokines, like IFNγ, might result from the diverse experimental settings or the intricate pathophysiological mechanisms of the TME. A certain cytokine, or one type of immune cell, could exert conflicting effects through distinct mechanisms regarding different stages of tumor development. For instance, IFNγ induces tumor cell apoptosis via its canonical JAK/STAT signaling pathway[219]. Conversely, it is reported that IFNγ plays an indispensable role in the inflammatory response that facilitates hepatocarcinogenesis at the initiation stage, involving immune cell activation and oxidative DNA damage induction, but not in the promotion stage[220]. Our results in this study have revealed IFNγ as a pro-inflammatory cytokine that is associated with a more aggressive GBM phenotype. IL-17 is a potent pro-inflammatory cytokine that amplifies the inflammatory response by stimulating massive cytokine production. Of note, IL-17 activates the IL-6/STAT3 signaling pathway and thus enhances tumor growth[221, 222]. Some glioma studies have revealed a positive correlation between IL-17 and tumor proliferation, migration and invasion[223, 224]. One clinical study suggested that IL-17 might be a beneficial prognostic indicator for GBM patients[225]. This conflicting result was obtained from relatively small sample recruitment(n=41) in one hospital. More evidence may be needed to support this perspective. IL-22 is particularly important in mediating the tumor-immune cell communication. Due to the absence of the IL-22 receptor on immune cells, T cell-secreted IL-22 can only act on non-hematopoietic cells, including tumor cells[226]. It has been suggested that IL-22 promotes GBM proliferation by activating the JAK/STAT signaling[227]. IL-10 is an essential anti-inflammatory and immunosuppressive cytokine that suppresses pro-inflammatory cytokine production and inhibits APC antigen presentation. It is reported that IL-10 suppresses the pro-

inflammatory Th17 cells and controls the tumor-promoting inflammation[228]. In general, our findings have not only verified the relation of these inflammatory cytokines and GBM malignancy, but also underlined the role of anakinra in regulating T cell cytokine production and thereby attenuating GBM aggressiveness.

4.3 Feasibility of anakinra in GBM treatment

Anakinra is a recombinant IL-1 receptor antagonist that has been approved to treat rheumatoid arthritis. Its off-label uses in various autoinflammatory diseases have also acquired beneficial therapeutic efficacies[229]. Clinical studies have provided a safety profile for anakinra administration, with a mild skin reaction as the most frequent adverse event[230]. Anakinra has a molecular weight of 17.3kD and a half-life of 4-6h. The short half-life of anakinra requires daily subcutaneous injection to maintain its therapeutic concentration, which is responsible for the prevalence of injection site reactions. However, it is better to manage adjustments with a short half-life, especially when immediate discontinuation is needed, which makes anakinra suitable for treating critical patients[231].

Although other IL-1 blockades with longer half-life are available on the market, such as rilonacept and canakinumab, they have much larger molecular weights that make it difficult to effectively penetrate through the blood-brain barrier[232]. Anakinra is able to cross the BBB and takes effects in the central nervous system. Multiple clinical studies have reported that anakinra has therapeutic benefits and neuroprotective effects in not only cerebral autoinflammatory disease[233], but also other pathological conditions in the brain as subarachnoid hemorrhage[234], cerebral ischemia[175] and epilepsy[235]. Even though this current study only examined the efficacy of anakinra in vitro experimental model, other evidence mentioned above has provided the feasibility for anakinra to treat GBM patients by suppressing the tumor-associated inflammation in the brain. Of course, future investigations on animal models and clinical studies will be needed to further validate the hypothesis.

Additionally, anakinra has shown favorable results in treating other types of cancer. For instance, anakinra reduces IL-22 production from T cells by abrogating IL-1 signaling and thereby inhibits tumor progression in a murine breast cancer model[236]. IL-1β inhibition by canakinumab has presented therapeutic potential in lung cancer treatment[237]. Our findings are compatible with these previous studies that targeting the inflammatory signaling in tumors using anakinra might be a promising option for cancer therapy.

4.4 Prospects

This study has demonstrated the efficacy of anakinra in alleviating GBM-associated inflammation and attenuating GBM malignancy, which provides a theoretical foundation for future research. Regarding the clinical use of anakinra, one study has reported that, for subarachnoid hemorrhage patients, the experimental effective concentration of anakinra in the CSF is 100ng/ml[234], which is much lower than the 1μg/ml applied in our study. We determined the 1μg/ml dosage of anakinra in these in vitro experiments due to its sufficient inhibition of IL-1β-induced inflammation. Our chosen concentration may not be able to directly apply in treating patients. Further in vivo experiments and clinical trials using different concentrations are indispensable to determine an optimal anakinra dosage for GBM therapy.

In the co-culture model of GBM and immune cells, we examined the impact of anakinra on T cells regarding their inflammatory markers and cytotoxic effector molecules. However, the innate immunity, especially the tumor-associated macrophages, also constitutes a crucial part of the GBM inflammatory microenvironment. It has been revealed that TAMs extensively contribute to GBM growth, metastasis, neoangiogenesis and the immunosuppressive microenvironment[238]. The effects of anakinra on TAM in the context of GBM has not been illustrated and worth to be investigated in future research.

4.5 Summary

In summary, the present study has validated the role of IL-1β in activating the inflammatory cascade in GBM that drives tumor development. Multiple pro-inflammatory cytokine expressions have been associated with GBM aggressiveness. The dynamic interaction between GBM and immune cells also induces a similar inflammatory response in GBM cells, as well as a more malignant phenotype with enhanced proliferation, migration and reduced apoptosis. Anakinra, a recombinant IL-1 receptor antagonist, can sufficiently dampen the inflammatory signaling in both GBM cells and T cells, and reverse the inflammation-associated GBM aggressiveness. Anakinra administration attenuates tumor progression by inhibiting proliferation, migration and inducing apoptosis. Therefore, anakinra has emerged as a promising therapeutic strategy to ameliorate GBM malignancy. Our study has provided the experimental foundation for future studies that target IL-1 signaling in GBM oncotherapy.

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Affidavit

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Tingting Wu

Signature doctoral candidate

List of publications

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