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The Potential for Genetically Altered Microglia to Influence Glioma Treatment

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Abstract

Diffuse and unstoppable infiltration of brain and spinal cord tissue by neoplastic glial cells is the single most important therapeutic problem posed by the common glioma group of tumors: astrocytoma, oligoastrocytoma, oligodendroglioma, their malignant variants and glioblastoma. These neoplasms account for more than two thirds of all malignant central nervous system tumors. However, most glioma research focuses on an examination of the tumor cells rather than on host-specific, tumor micro-environmental cells and factors. This can explain why existing diffuse glioma therapies fail and why these tumors have remained incurable. Thus, there is a great need for innovation. We describe a novel strategy for the development of a more effective treatment of diffuse glioma. Our approach centers on gaining control over the behavior of the microglia, the defense cells of the CNS, which are manipulated by malignant glioma and support its growth. Armoring microglia against the influences from glioma is one of our research goals. We further discuss how microglia precursors may be genetically enhanced to track down infiltrating glioma cells.

Keywords

Glioblastoma; M2 polarization; microglia; pathway analysis; systems biology; zinc finger nucleases

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

INTRODUCTION

Gliomas are tumors derived from glial cells, the non-neuronal cells of the brain and spinal cord. They are the most common tumors of the central nervous tissue and many are fatal comprising more than two thirds of all malignant CNS neoplasms. Gliomas occur in adults as well as children, and their prognosis depends on subtype and grade (www.pubcan.org). A tentative diagnosis can be made by means of modern neuroimaging techniques but the gold standard for glioma diagnosis is the microscopic examination of tumor tissue following a surgical biopsy. Currently, the microscopic examination can be complemented but not replaced by molecular tests. Therefore, only a trained neuropathologist should make the diagnosis by morphologic criteria. This is important because other CNS diseases can present with tumor-like signs. An accurate diagnosis is of key importance for the decision on treatment strategies. Surgery as well as radio- and chemotherapy may be applied.

The vast majority of contemporary glioma research focuses on the tumor as such rather than on what the central nervous system (CNS) does or does not do to the infiltrating neoplastic glial cells. This can explain why all existing diffuse glioma therapies fail and why the prognosis of these tumors has changed insignificantly in more than a century and they have remained incurable. Consequently, there is a great need for innovation. Recent advances in neuroscience knowledge and technological progress in molecular biology raise hope that such innovation is within reach.

In this article we outline a novel strategy that focuses on making use of the presence of microglia within diffuse glioma for therapeutic purposes. There is increasing evidence that high-grade gliomas very effectively attract microglia/macrophages and subsequently control their activity eliciting mainly tumor-supportive functions that facilitate glioma growth [1]. We are interested in the question of whether this fatal attraction can be used against the tumor by employing bone-marrow transplantation of genetically enhanced [2] microglia precursors. We further discuss the need for the development of an *in silico* model of the microglia, which is expected to yield a blueprint of the molecular controls that are required to modify the behavior of glioma associated microglia. In addition, our vision for engineering microglia that are capable of tracking down individual, deeply infiltrating glioma cells is outlined. Lastly, we describe a technology known as zinc finger nucleases (ZFNs) that may be employed to implement the required genetic modifications.

THE CURRENT GOLD STANDARD OF GLIOMA DIAGNOSIS

The microscopic examination of a tumor tissue reveals the histological tumor type. Gliomas are named after the normal glial cell types with which each tumor variant shares morphological similarities. The two main neuroglial cell types of the CNS are astrocytes and oligodendrocytes. The third common glial cell type, the microglia, populate the CNS during embryonic and early postnatal development and cause tumors so rarely that there is no official classification entry [3]. After the type of brain tumor has been determined based on morphological criteria, a WHO grade is assigned. The WHO classification of tumors (www.pubcan.org) currently distinguishes the main subtypes of common glioma shown in Table 1.

In general terms, a tumor is referred to as grade I if the biopsy shows only very few dividing cells and the chances of the patient is being cured by the surgical resection of the tumor alone are high. A grade II tumor in contrast is likely to recur and may even worsen over time, i.e. become anaplastic. The latter is called tumor progression and is regularly the case for diffuse astrocytoma, oligoastrocytoma and oligodendroglioma. Histological signs of a malignant or WHO grade III glioma include so-called atypical nuclei and the presence of dividing cells (mitosis). Patients with grade III tumors are treated by adjuvant radiation and/or chemotherapy. Glioblastoma is an example of a WHO grade IV tumor. In addition to cells that appear malignant and variable numbers of mitotic cells, such tumors tend to show large areas of cell death (necrosis) because they grow so rapidly that the blood supply cannot keep up with their growth rate. However, grade IV tumors also stimulate the formation of new blood vessels (neoangiogenesis) and the presence of the latter is another and perhaps even more significant histological sign of their malignancy.

In summary, the WHO classification of CNS tumors (www.pubcan.org) represents a malignancy scale aimed at aiding the clinician to choose the right treatment. It is not always strictly logical. For instance, there is no pilocytic astrocytoma grade II and there is no diffuse astrocytoma grade I. These two entities are very different biologically but share the common family name astrocytoma. Patients with a WHO grade II glioma usually survive more than five years whereas survival of 2–3 years are typical for an individual with a WHO grade III tumor. The outcome is much worse for WHO grade IV glioblastoma where less than half the patients survive more than one year.

THE PRESENCE OF MICROGLIA AND RELATED CELLS WITHIN DIFFUSE GLIOMA

The occurrence of microglial cells in glioma is not a new finding [4, 5]. However, their role remained unclear for many decades. In 1998 we reported that microglia support glioma growth [6]. This finding has been widely reproduced in the meantime and microglia research has yielded much information on the molecular characteristics of these cells. In 1998 we also demonstrated that bone marrow-derived precursors can give rise to typical ramified parenchymal microglia in the adult [7, 8], a concept now adopted by others [9]. These observations are of great relevance in the context of our plan to send genetically enhanced microglia as therapeutic agents into diseased brains. It is likely that many if not most of the glioma-associated macrophages and microglia (ramified cells of typical morphology with perpendicularly branching cell processes) have an extra-cerebral source. The phenotypic and functional differences between these largely “M2 polarized” cells and classically activated (inflammatory) “M1 macrophages” [10] are intriguing and point to a strategy gliomas employ to manipulate microglial behavior in favor of tumor survival and growth.

Analyses of the signaling networks by which microglia interact with the glioma suggests the following view [1]: Glioma-microglia synergies drive a self-amplifying cascade of events that spirals out of control as the tumor progresses. Glioma and microglial cells not only appear to have a symbiotic relationship but one that becomes highly skewed in favor of the glioma [11]. Specifically, the immunosuppressive microenvironment in a glioma created by molecules such as TGFB1, CSF1, and IL10 polarizes glioma-infiltrating microglia towards

the M2 phenotype. Gliomas also produce chemotactic factors, such as MCP-1, resulting in the recruitment of large numbers of additional microglia and macrophages. Gliomas further promote the proliferation of microglial cells. In turn, microglia support glioma angiogenesis as well as glioma cell invasion. This cross talk between glioma and microglia is governed by multiple paracrine loops formed by glioma- and microgliareleased molecules and their receptors. Some of the molecules involved also act in an autocrine manner regulating glioma and microglia behavior, respectively (for details see [1]). It therefore appears that immune cells, which are a major source of angiogenic and growth factors as well as matrix-remodeling enzymes that have an entirely normal and necessary function in wound healing, are recruited and subverted to support neoplastic progression [12] in glioma.

There is an additional and particularly interesting aspect of microglia-tumor cell interactions that has attracted attention only very recently. Fusion of tumor cells with bone marrow-derived cells has been proposed as a mechanism underlying invasion and metastasis in human cancer [13]. Accordingly, cellular fusion of microglia with glioma cells is being considered as a possible explanation for the surprising finding that isocitrate dehydrogenase mutations can be observed in microglia/macrophages associated with glioma [14]. We have suggested earlier that some macrophages in glioblastoma may derive from tumor stem cells [15], which would be in line with the view of a significant role of microglia/macrophages in glial tumorigenesis [14]. The fusion hypothesis is of particularly great interest as macrophages are highly migratory cells and gliomas are the most diffusely growing tumors of all. One way to test this hypothesis experimentally will be to use gender-mismatched microglia and glioma cells in combination with FISH to detect Y-chromosomal sequences in cells exhibiting a macrophage phenotype (the tumor cell line, e.g. CNS-1, being derived from male animals).

Taken together, the microglial contributions to glioma growth appear significant and justify serious efforts to study microglia/macrophage-glioma interactions in the greatest detail possible and not only to bring genetically modified microglia into the glioma-affected brain to exert an inhibitory influence on glioma growth but to reduce the vulnerability of the microglia towards glioma influences as the first step.

NON-INVASIVE ACCESS TO THE CNS

Using bone-marrow chimeras carrying a non-expressed marker gene and a combined model of facial nerve axotomy and transfer experimental autoimmune encephalitis, we demonstrated that cells from the macrophage precursor cell pool of the bone marrow have the ability to become typical ramified microglia in the adult [8]. Thus, if recently bone marrow-derived parenchymal microglia fully integrate into a regenerating brain nucleus' architecture, entirely new approaches for delivering genes into the adult CNS become a possibility [8]. The validity of this hypothesis has been dramatically confirmed by the recent finding that pathological grooming in Hoxb8 mutant mice can be cured through a bone marrow transplant [16]. Furthermore, transplantation of wild-type bone marrow into irradiation-conditioned Mecp2-null hosts resulted in engraftment of brain parenchyma by bone-marrow-derived myeloid cells of microglial phenotype, arresting the development of disease [17]. Importantly, only the use of a conditioning regimen capable of ablating

functionally defined brain-resident myeloid precursors allows turnover of microglia that is mediated by local proliferation of early immigrants rather than entrance of mature cells from the circulation [18].

We are using a syngeneic glioma model that employs GFP-transgenic Lewis rats and mCherry-transduced CNS-1 [19, 20] glioma cells (Fig. 1). Our goal is to achieve non-invasive access to the experimental glioma through autologous bone marrow-transplants. As a first step, we are replicating earlier results from a C6 glioma model, which based on use of a non-expressed marker gene strongly suggested that a significant portion of the microglia/macrophages in glioma are bone marrow-derived [21].

TOWARDS AN *IN SILICO* MODEL OF MICROGLIAL CELLS

There is currently no complete systems biological definition of microglial cells (or of any other cell type). Starting out from a first partial transcriptome signature, which we obtained previously [22], we are currently complementing our database by mining publicly available microarray datasets. Pathway analysis software [23, 24] is used to extract information that can assist with the design of an *in silico* microglia pathway model, which will serve as a blueprint for the controls of microglial behavior *in vivo*. Reducing microglial susceptibilities to the influence of glioma (e.g. by knocking out receptors such as IL4R, Table 2, Figs. 1, 2) while strengthening or introducing other properties, e.g. the ability to track down glioma cells similar to what stem cells can do [25–32], represent key areas where this *in silico* knowledge will be applied.

NEW MOLECULAR GENETIC METHODS FOR REPROGRAMMING CELLS

Technologies with the potential for editing the genome hold great promise for cell-based therapies. Termed zinc finger nucleases (ZFN), one such technology, which has matured significantly in recent years, combines the most abundant DNA binding motif, zinc fingers and the power of restriction endonucleases to provide sequence-specific modification of the genome. Zinc finger proteins (ZFPs), discovered in Xenopus the mid-1980's [33], are the largest class of DNA-binding proteins found in eukaryotic cells. They serve diverse roles in most cell processes including DNA replication and repair, transcription, translation, metabolism and cell signaling among others [34]. Each zinc finger motif consists of approximately 30 amino acids folded into a ββα structure. A ZFP recognizes three bases in a DNA sequence *via* the single α-helical structure in the C-terminal region of the protein and binds by inserting the α-helix into the major groove of the DNA double helix [35]. The stability of the entire protein complex is afforded by the anti-parallel β-β hairpin structure present at the N-terminal region. The hairpin is created by the binding of a Zn^{2+} ion to two canonical cysteine residues that are generally 2–4 amino acids apart followed by the zinc ion interaction with two histidine residues, commonly referred to as the C_2H_2 zinc finger [36]. The discovery that several ZFPs linked in tandem are capable of recognizing a broad spectrum of DNA sequences with high specificity opened a "toolbox" capable of tinkering with the molecular machinery of a cell.

In 1996, a report on the generation of a fusion construct between zinc finger proteins and the nuclease domain of the non-discriminative, type IIS restriction enzyme FokI heralded a new

era in DNA manipulation [37]. The uniqueness of this design was many-fold. The first among these was the ability to engineer tandem ZFPs to target specific DNA sequences. This implied that by linking engineered ZFPs in tandem, it was technically possible to target any DNA sequence in the human genome – a highly sought after molecular tool with the ability to manipulate the human genome at desired sites. Secondly, the use of the FokI restriction nuclease implied that enzymatic activity would only be present when the cleavage domain was present as a dimer, an intrinsic characteristic of the enzyme [38]. This empowers the ZFP-FokI hybrid, commonly referred to as a zinc finger nuclease (ZFN), with further specificity as each half of the nuclease dimer is fused to ZFPs flanking the desired cleavage site in DNA. Additionally, since the ZFPs bind to opposite strands of the DNA, the ZFN creates a highly desirable double-strand break (DSB) at the target locus in the genome. It is well established that cells employ the universal process of homologous recombination (HR) to mediate site-specific recombination following DSB in DNA in order to maintain genomic stability and integrity. This phenomenon offers another advantage to the ZFN technology whereby “correction” of the cleaved DNA helix can be afforded by introducing a targeting DNA sequence homologous to the cleaved segment but bearing the “corrected” or “edited” gene sequence. DSB repair of damaged DNA by HR is the most accurate form of cellular repair that usually employs the undamaged sister-chromatid as a template.

ZFN technology is gaining more wide use. Investigations in mammalian as well as other systems have revealed the key parameters that offer maximum efficacy of targeting. Continuous minor modifications are honing the technology. A plethora of studies have demonstrated the potential of the technology and clinical trials are underway [39–46]. We are planning to apply this technology to the genetic modification of microglial cells and their precursors.

SUMMARY

The clinical consequences of diffuse glioma are serious and their prognosis is dire. Symptoms range from neurological and other somatic deficits to cognitive and psychological problems. As a result, brain tumors cause the fourth highest loss of potential life years of all cancers. This justifies an intense research effort. Importantly, after decades of failure there is a clear case for more interdisciplinary research and specifically studies into the question of what CNS constituents do and do not do in support of glioma cell growth. By combining experimental neuropathological and immunological with some of the latest molecular genetics techniques, the approach outlined here will contribute to an improved understanding of bone marrow-derived microglia and their suitability for the treatment of CNS disorders. It will be tested using glioma as a first target. There is reason for optimism because successful cell-based treatments for nervous system disorders employing bone marrow-transplantation are already beginning to emerge in other areas [47]. If successful the results of the work proposed here are likely to be of relevance also for other cancers that are characterized by the presence of macrophages [48]. ZFNs and new methods such as TALENs that are currently being developed [49] are expected to facilitate the synthetic biological engineering of microglia precursors and will assist in making use of the microglia as a novel and powerful vehicle for treating glioma. Ultimately such engineered

cells could also be made to carry a payload [50, 51] that may be of additional diagnostic as well as therapeutic utility through interactions with hadrons for instance [52] (Fig. 3).

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ABBREVIATIONS

ADAM8	ADAM metallopeptidase domain 8
AKT1	v-akt murine thymoma viral oncogene homolog 1
CCL2	Chemokine (C-C motif) ligand 2
CD4	CD4 molecule
CD14	CD14 molecule
CD40	CD40 molecule, TNF receptor super family member 5
CD40LG	CD40 ligand
CD86	CD86 molecule
CD8A	CD8a molecule
CNR1	Cannabinoid receptor 1
CNS-1	Glioma cell line syngeneic to Lewis rats
CNS	Central nervous system
CSF1	Colony stimulating factor 1 (macrophage)
CSF1R	Colony stimulating factor 1 receptor
CSF2	Colony stimulating factor 2 (granulocyte-macrophage)
CSF2RA	Colony stimulating factor 2 receptor, alpha, low-affinity (granulocyte-macrophage)
DNA	Deoxyribonucleic acid
DSB	Double-strand break
EGFR	Epidermal growth factor receptor
FAS	Fas (TNF receptor super family, member 6)
FISH	Fluorescent in situ hybridization
FOKI	A restriction endonuclease
IFNB1	Interferon, beta 1
IFNG	Interferon, gamma
IFNGR2	Interferon gamma receptor 2 (interferon gamma transducer 1)
IFNGR1	Interferon gamma receptor 1

IL1B	Interleukin-1, beta
IL4	Interleukin-4
IL4R	Interleukin-4 receptor
IL6	Interleukin-6 (interferon, beta 2)
IL6R	Interleukin-6 receptor
IL10	Interleukin-10
IL10RA	Interleukin-10 receptor, alpha
IL12	Interleukin-12
IL13	Interleukin-13
MAPK1	Mitogen-activated protein kinase 1
MAPK14	Mitogen-activated protein kinase 14
MAPK3	Mitogen-activated protein kinase 3
MAPK8	Mitogen-activated protein kinase 8
MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)
NGF	Nerve growth factor (beta polypeptide)
NOS2	Nitric oxide synthase 2, inducible
PTPN6	Protein tyrosine phosphatase, non-receptor type 6
RAF1	v-raf-1 murine leukemia viral oncogene homolog 1
REL	v-rel reticuloendotheliosis viral oncogene homolog
SOCS1	Suppressor of cytokine signaling 1
SOCS3	Suppressor of cytokine signaling 3
STAT1	Signal transducer and activator of transcription 1
STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)
TGFB1	Transforming growth factor, beta 1
TGFBR2	Transforming growth factor, beta receptor II (70/80kDa)
TLR4	Toll-like receptor 4
TNF	Tumor necrosis factor
TNFRSF1A	Tumor necrosis factor receptor super family, member 1A
TP53	Tumor protein p53
ZFN	Zinc finger nucleases
ZFP	Zinc finger protein

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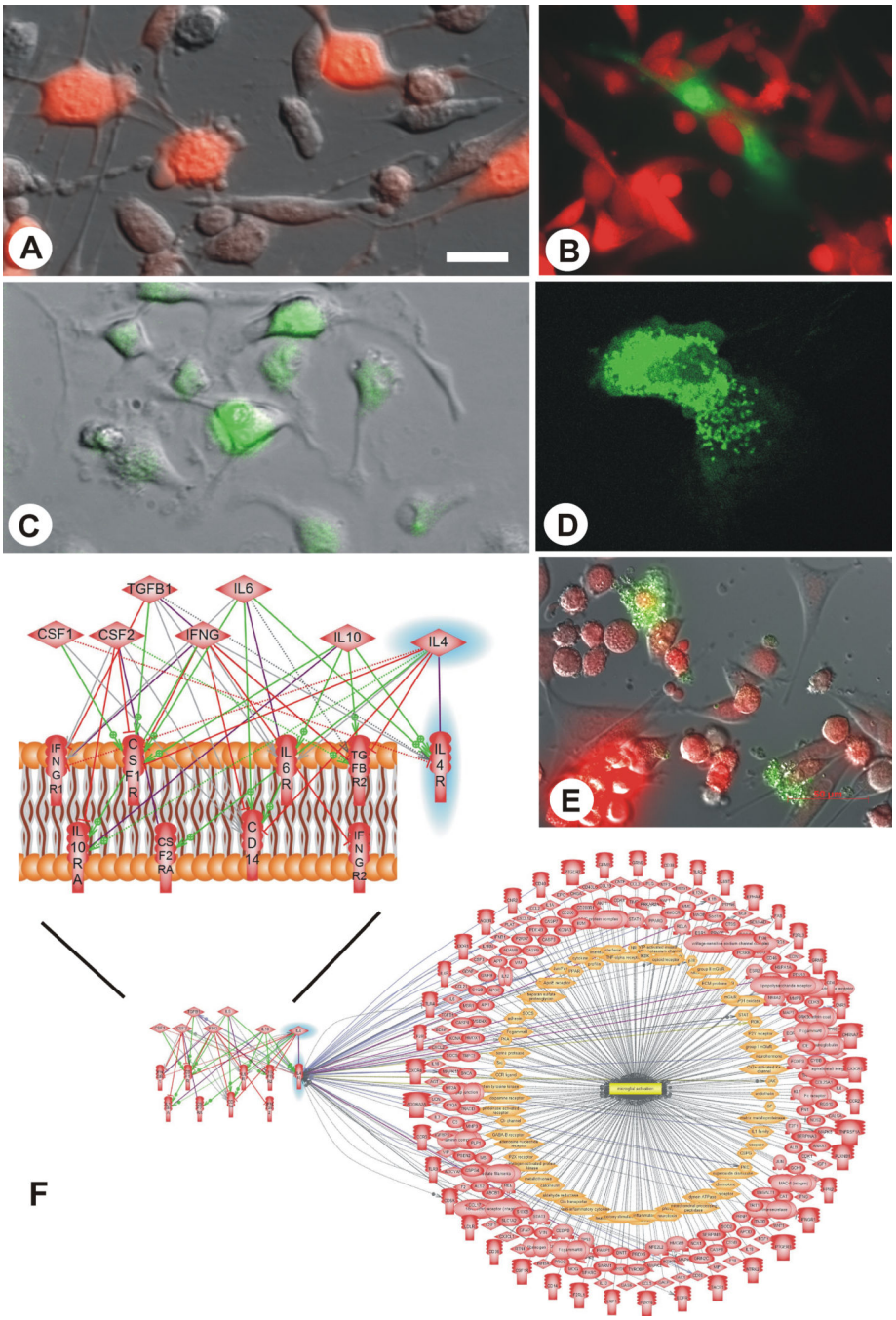

Fig. (1).

Experimental tools used in the project. **A**. mCherry labeled CNS-1 glioma cells in culture; **B**. transgenic astrocytes (green, obtained from GFP-transgenic Lewis rats) being overrun in culture by syngeneic mCherry labeled CNS-1 glioma cells (red) mirroring the *in vivo* situation; **C**. bone marrow cells isolated from GFP-transgenic Lewis rats in culture; **D**. a GFP-transgenic microglial cell/macrophage in culture; **E**. Co-culture of mCherry labeled CNS-1 glioma cells and microglia isolated from GFP transgenic rats; **F**. schematic representation of intercellular microglia “polarization signaling”: CSF2 (GM-CSF) and

IFNG are the molecules that drive microglia towards the M1 phenotype, whereas TGFB1, CSF1, IL10, IL4 and IL6 are molecules that polarize microglia towards the M2 phenotype when binding to their respective receptors located on the surface of microglia (purple lines); however, these signals may also act on other receptors on the surface of the microglia; stimulatory regulation is represented by green lines, inhibitory regulation by red lines, and ambiguous effects are shown as grey lines; IL-4 and its receptor, IL-4R are highlighted in blue; the biological associations of the latter with molecules that are regulated during microglial activation are illustrated by the lower panel of this figure and referenced in detail in Table 2. Scale bar: 40 μm (appx. 80 in E).

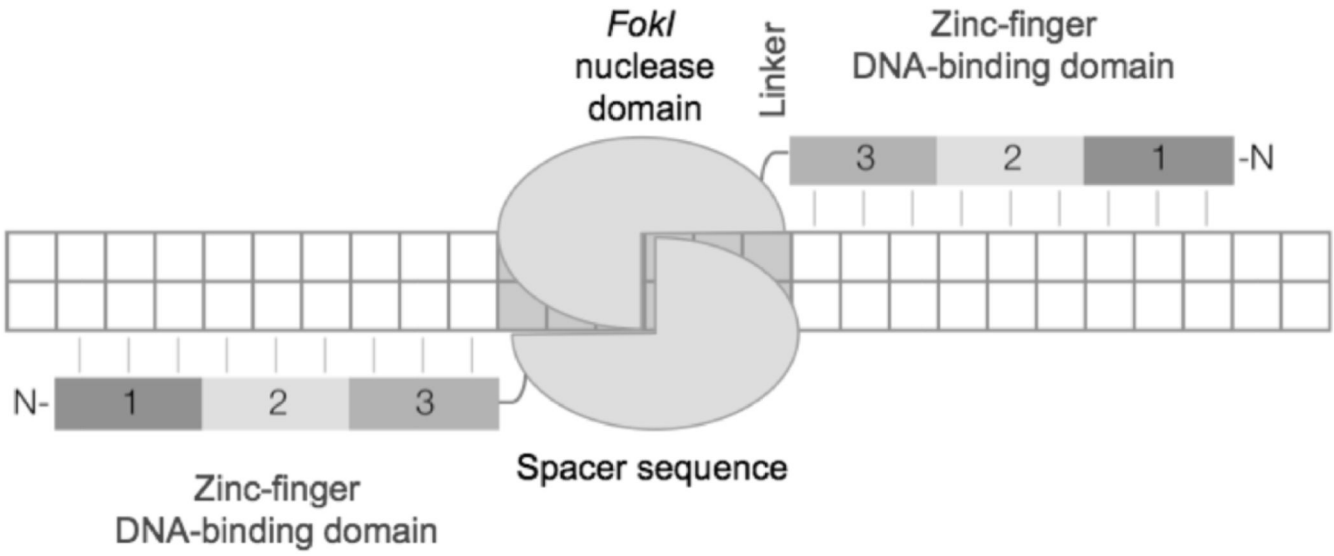

Fig. (2).

Zinc finger nuclease (ZFN)-mediated DNA double-strand break. A ZFN designed to create a DNA double-strand break (DSB) in the target consists of two monomers. Each monomer encompasses three zinc-fingers (1, 2, 3), which recognize 9 base pairs within the target and a FokI nuclease domain. A short “linker” sequence connects the two domains. The FokI nuclease only functions as a dimer and therefore, following dimerization the nuclease is activated and cleaves the DNA within the spacer sequence.

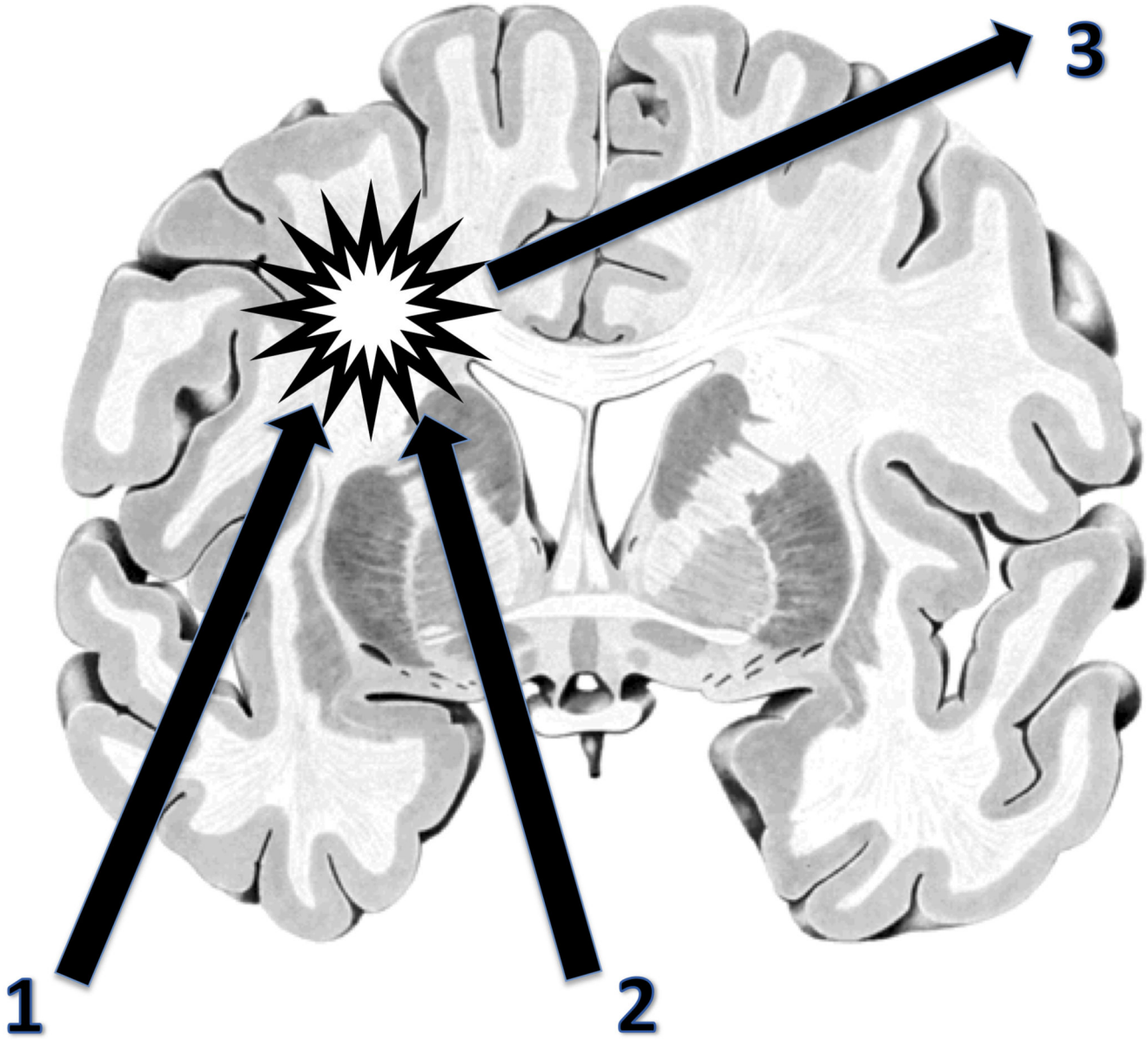

Fig. (3).

Vision for a future glioma treatment utilizing the potential of genetically enhanced microglia: two non-invasive strategies converging on the tumor. **1**. Once the trafficking (entry into the CNS) and tracking (following glioma cells) behavior of the microglia can be controlled satisfactorily, these cells like other macrophages could also be employed to carry a payload [50, 51]. **2**. Very precise and effective and therefore perhaps even curative tissue destruction/stimulation could then be achieved by means of a hadron beam [52]. **3**. This approach may also allow real-time monitoring of the treatment if appropriate radiation sensors are implanted stereotactically (initially, for calibration purposes) and suitable radiochemicals for visualization become available. The glioma and its diffusely infiltrating

cells is represented by the star. Coronal brain slice taken from inter BRAIN 1.1 for Windows, Springer 1998.

Table 1

Main Types of Human Glioma

Astrocytic tumors
Pilocytic astrocytoma (I)
Pilomyxoid astrocytoma (II)
Subependymal giant cell astrocytoma (I)
Pleomorphic xanthoastrocytoma (II)
Diffuse astrocytoma (II)
Fibrillary astrocytoma
Gemistocytic astrocytoma
Protoplasmic astrocytoma
Anaplastic astrocytoma (III)
Glioblastoma (IV)
Giant cell glioblastoma
Gliosarcoma
Gliomatosis cerebri (III)
Oligodendroglial tumors
Oligodendroglioma (II)
Anaplastic oligodendroglioma (III)
Oligoastrocytic tumors
Oligoastrocytoma (II)
Anaplastic oligoastrocytoma (III)
Ependymal tumors
Subependymoma (I)
Myxopapillary ependymoma (I)
Ependymoma (II)
Cellular
Papillary
Clear cell
Tanycytic
Anaplastic ependymoma (III)

Grades of the different glioma variants are indicated by Roman numerals shown in brackets following the respective glioma subtype.

Table 2

Biological Associations Relating to the IL4R Receptor

Relation	Type	Reference
CD4 ---> IL4R	Regulation	[53, 54]
CD40 ---> IL4R	Expression	[55–58]
CD40LG --+> IL4R	Expression	[59, 60]
CD86 --+> IL4R	Regulation	[61]
IFNB1 ---> IL4R	Expression	[62]
IL12 --+> IL4R	Expression	[63–66]
IL13 --+> IL4R	Direct Regulation	[67–165]
IL1B ---> IL4R	Expression	[166–168]
IL4R --+> CD8A	Regulation	[169–171]
IL4R --+> CNR1	Expression	[172]
IL4R --+> NGF	Molecular Transport	[173]
IL4R --+> STAT3	Regulation	[174, 175]
IL4R ---- PTPN6	Binding	[176–181]
IL4R ---- SOCS3	Binding	[182]
IL4R ---- TP53	Binding	[183]
IL4R ---> ADAM8	Expression	[184]
IL4R ---> AKT1	Regulation	[185, 186]
IL4R ---> CCL2	Expression	[187]
IL4R ---> CD4	Regulation	[188–195]
IL4R ---> CD86	Expression	[57, 61, 196]
IL4R ---> EGFR	Regulation	[197]
IL4R ---> IL12	Expression	[95, 198, 199]
IL4R ---> IL1B	Regulation	[83]
IL4R ---> MAPK14	Regulation	[200]
IL4R ---> MAPK3	Regulation	[156, 201]
IL4R ---> MAPK8	Regulation	[156]
IL4R ---> STAT1	Regulation	[202, 203]
IL4R ---| FAS	Regulation	[204, 205]
IL4R ---| IFNGR1	Regulation	[202]
IL4R ---| MAPK1	Regulation	[206]
IL4R ---| NOS2	Regulation	[90]
IL4R ---| TLR4	Regulation	[207]
MAPK1 --+> IL4R	Regulation	[208]
MAPK14 --+> IL4R	Expression	[209]
MIF --+> IL4R	Regulation	[210]
RAF1 ---> IL4R	Regulation	[211]
REL ---| IL4R	Expression	[212]

Relation	Type	Reference
SOCS1 ---| IL4R	Regulation	[213]
STAT1 ---> IL4R	Expression	[214]
TNF --+> IL4R	Expression	[215–219]
TNFRSF1A ---> IL4R	Regulation	[217]

--+>Positive influence.
---|Negative influence.
(Pathway Studio, Elsevier).