Abstract: Toll-like-receptor (TLR) family members were detected in the central nervous system (CNS). TLR occurrence was noticed and widely described in glioblastoma-multiforme (GBM) cells. After ligand attachment, TLR-4 reorients domains and dimerizes, activates an intracellular cascade, and promotes further cytoplasmatic signaling. There is evidence pointing at a strong relation between TLR-4 signaling and micro ribonucleic acid (miRNA) expression. The TLR-4/miRNA interplay changes typical signaling and encourages them to be a target for modern immunotherapy. TLR-4 agonists initiate signaling and promote programmed death ligand-1 (PD-1L) expression. Most of those molecules are intensively expressed in the GBM microenvironment, resulting in the autocrine induction of regional immunosuppression. Another potential target for immunotreatment is connected with limited TLR-4 signaling that promotes Wnt/β-catenin signaling, resulting in a limitation of GBM invasiveness. Interestingly, TLR-4 expression results in bordering proliferative trends in cancer stem cells (CSC) and GBM. All of these potential targets could bring new hope for patients suffering from this incurable disease. Clinical trials concerning TLR-4 signaling inhibition/promotion in many cancers are recruiting patients. There is still a lot to do in the field of GBM immunotherapy.

Keywords: glioblastoma multiforme; TLR; TLR-4; toll-like receptor; glioma; high-grade glioma

1. Introduction

Various toll-like-receptor (TLR) family members are detected in the central nervous system (CNS). They are mainly expressed in neurons and glial structures, where they play a role in recognizing unfamiliar molecules, some postapoptotic antigens consequently control repair processes and modulate inflammatory action [1].

Furthermore, TLR occurrence was noticed and widely described in glioblastoma-multiforme (GBM) mature cell fractions [2]. Studies revealed a strong expression of these receptors in cohorts of neural non-pro-oncogenic stem cells [3,4]. GBM, as the most popular and expansive glioma form, with a mean survival of 14.6 months, remains a challenge for modern therapy [5]. The characteristics
of TLRs make them a promising target for GBM immunotreatment. Activated receptors stimulate the response of the immune system and control the course of many diseases, including cancers [6–9]. The necrotic ratio during glioblastoma invasion remains high. Products of cell breakdown intensively interact with the transmembrane architecture of TLRs and promote differentiation and inflammatory signaling [10]. In brain tissue, TLR-4 is detected in two main types of cells: microglial (macrophages) and macroglial cells (oligodendrocytes and astrocytes). Microglia do not intensively express TLR-4 on the surface (less than 15%), and receptors are easier to detect intracellularly. Macroglial cells superficially express TLR-4. Oligodendrocytes and astrocytes do not intracellularly express TLR-4 at all. This difference in expression could be explained by the different phagocytic functions of micro- and macroglial cells (Table 1) [11,12].

Table 1. Toll-like-receptor (TLR)-4 expression in brain tissue, cancer lines, and tumors.

| mRNA/protein  | Localization   | Type of Cells                     |
|--------------|----------------|-----------------------------------|
| Tissue       | Neurons, microglia [13–15] |
| Tissue       | Astrocytes [16,17] |
| mRNA         | Cell Lines     | Glioma (U87, SF126, U251, GI 261) [18] |
| mRNA/protein | Tumor/Cell Lines | Astrocytoma/GBM (U87MG, A172, LN229, U118) [19] |

The TLR family is a group of ten receptors (TLR-1–TLR-10) characterized by the detection of a particular pattern of micro-organisms (pathogen-associated molecular patterns (PAMPs)), which are invariant for most pathogens and not present in mammalian organisms. TLR receptors are also sensitive to particles secreted during necrosis and cell death called danger-associated molecular patterns (DAMPs) [20–23]. Typically, TLRs are grouped into two major categories, endosomal (TLR-9, TLR-8, TLR-7, and TLR-3) and cell-surface-acting (TLR-10, TLR-6, TLR-5, TLR-4, TLR-2, and TLR-1) [24–26]. TLRs functioning endosomally are mainly activated with nucleic acids. On the other hand, a variety of molecules activate TLRs expressed on the cell surface. Most of them include lipoproteins. After ligand attachment, TLRs reorient domains and dimerize, activate intracellular cascade, and promote further cytoplasmatic signaling [27,28]. Immunotherapeutic agents aim at this activation chain, targeting immune-related disorders. Many autoregulating mechanisms control inflammation that is mediated through TLR signaling. Their activity concerns the nucleus level (countering the expression of cytokines and interleukins (TTP, ATF3, and REG-1) and the cytoplasmatic level (STAT1, AhR, Nurrl); the inhibition of adaptor complex suppressor of cytokine signaling, sterile alpha-and armadillo-motif-containing protein (SOCS1, SARM); and the cell-surface level interrupting dimerization processes: suppressor of tumorigenicity 2, single immunoglobulin IL-1R-related molecule, Rickettsia prowazekii 105 (ST2, SIGGR, and RP105) [29–33]. Moreover, some studies revealed microRNA molecules over activity destabilizing the mRNA of various cytokines. MiR-155-5p targets the MyD88 complex, similarly to MiR 203-5p and MiR 149-5p [30,34]. The described mechanisms of autocontrol ensure an adequate reaction to microbe-associated molecular patterns (MAMPs) and danger-associated molecular patterns (DAMPs), protecting autoimmunity and excessive response. TLR-4, after particular antigen attachment to a pathogen-associated molecular patterns (PAMP) or DAMP, is shifted from the cell surface to form the endosome during phagocytosis (Table 2).
Table 2. Characteristics of TLR-4 receptor: types of cells expressing TLR-4, pathogen-associated molecular patterns (PAMPs), danger-associated molecular patterns (DAMPs), and clinical-trial agents.

| TLR-4 | Types of Cells | PAMPs | DAMPs | Clinical-Trial Agent Characteristics |
|-------|----------------|-------|-------|--------------------------------------|
|       | Monocytes      |       | HSPs, heparin, fibrinogen, fibronectin, sulfate, HMGB1, ANG II | Anti-TLR-4 antibody, lipid A derivates, polysaccharides. |
|       | Macrophages    |       |       |                                       |
|       | Neutrophil     |       |       |                                       |
|       | Myeloid dendritic cells | Lipopolysaccharide |       |                                       |
|       | Mast cells     |       |       |                                       |
|       | B cells        |       |       |                                       |
|       | Intestinal epithelium |       |       |                                       |
|       | Platelets      |       |       |                                       |

2. TLR-4 Overview

Toll receptors (TRs) were detected in drosophila embryos by Hashimoto et al. (1988) [35,36]. Hoffmann and their study team proved that TR mutation in drosophila raised the risk of fungal infection. The above experiments indicated that the innate immune response has the ability to precisely detect the invasion of bacteria and other micro-organisms [37]. Further studies identified mammalian TR homologs that researchers called toll-like receptors (TLRs). The first, discovered by Medzhitov R. and colleagues in 1997, was named TLR-4 [38,39]. The TLR-4 gene is localized on the SSC9:119.5, 9q32-33 chromosome, structured with three exons. The receptor is built of four domains and 838 amino acids. The first, the extracellular, consists of 624 amino acid molecules; the second, the transmembrane, consists of 33 amino acids; the third, the cytoplasmatic proximal, of 159 amino acids; and the cytoplasmatic distal of 19 amino acids. The ectodomain is built of 21 leucine-rich repeat regions (LRRs), part of the extracellular domain [40–42]. TLR-4 presents multiple polymorphism phenomena concerning single nucleotides (SNPs). Most of them, recognized in the ectodomain, promote detrimental phenotypic outcomes [43,44].

2.1. TLR-4 Signaling

To bind lipopolysaccharides, TLR-4 requires co-operating molecule myeloid differentiation 2 molecule (MD-2), extracellularly stabilizing the ligand during activation. After antigen ligation, the receptor dimerizes and activates TIR domains; to properly signal, TLRs require MyD88, an essential adaptor activated during the first stages after pattern ligation to promote an immune response. Activation of the transmembrane receptor structure of TLR-4 transfers signaling in two major ways, the TIRAP–MyD88-dependent pathway controlling primary nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) promotion, and the combined production of inflammatory cytokines. Activation of MyD88 results in IL-1R-associated kinase 1/TNFR-associated factor 6 (IRAK-1/TRAF-6) induction with collateral ubiquitination, and TRAF-6 promotes Transforming growth factor-beta-activated kinase 1 (TAK-1). Activation of TAK-1 results in the formation and promotion of the IKK/NF-κB complex. Mitogen-activated protein kinases-c-Jun. N-terminal kinase (MAPK-JNK) and extracellular signaling-regulated kinase (ERK1/2) kinases are also activated, which indicates activated protein-1 (Ap-1). Downsignaling promotes transcription factors, such as activated Ap1, stabilizing many genes with regulatory properties during inflammation [45–47].

The second toll-like receptor adaptor molecule-1-translocating chain-associating membrane (TRIF-TRAM) signaling pathway (MyD88-independent) activates interferon regulatory factor-3 (IRF-3). TRAM signaling, through TRIF activation, results in TRAF-3 and TRAF-6 promotion (Figure 1); RIP is recruited by TRAF-6, and RIP signaling is activated on TAK-1/ERK1/2/Ap-1 axis. TRAF-3 activates IKK and TBK-1 to activate IRF-3. IRF-3 as a transcription factor that upregulates genes, coding mainly the first types of IFNs and other proinflammatory cytokines. The MyD88-independent pathway...
stimulates TNFα secretion and production. The sequential ligation of TNFa to a proper receptor favors NF-κB promotion. Therefore, the MyD88-independent pathway induces the activation of NF-κB in a late phase via IRF-3 and the secretion of TNFα. Proinflammatory molecules, such as IFN, pro-IL-1, and pro-IL-6, are also promoted [48–52].

**Figure 1.** Toll-like-receptor (TLR)-4, after activation by pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) supported by myeloid differentiation factor-2 (MD-2), initiates downstream signaling in two major ways. The first is the MyD88-dependent pathway connected with toll-interleukin 1 receptor domain-containing adaptor protein (TIRAP) resulting in myddosome formation, a structure containing IRAK-4 and IRAK-1 kinases. Activation of IRAK-1 results in TRAF-6 induction with collateral ubiquitination and phosphorylation. TRAF-6 promotes TAK-1. Activation of TAK-1 results in IKK/NF-κB complex formation and promotion. ERK1/2 kinases are also activated, which indicates activated protein-1 (Ap-1), the factor activating the transcription process. The second pathway, MyD88-independent, connected with translocating chain-associating membrane (TRAM), leads through toll-like receptor adaptor molecule-1 (TRIF) activation resulting in TRAF-3 and TRAF-6 promotion. RIP is recruited by TRAF-6, activating RIP signaling on the TAK-1/ERK 1/2/Ap-1 axis. TRAF-3 activates IKK and TBK-1 to activate interferon regulatory factor-3 (IRF-3). IRF-3 induces interferon expression and other proinflammatory cytokines.
2.2. Progression of GBM

Numerous signaling pathways induce carcinogenesis, oncogenic transformation, and invasiveness of cancer cells. Interestingly, some of them promote NF-κB expression. NF-κB, as an important transcription factor, is involved in various cellular processes, including migration, proliferation, and survival. Inflammatory cytokines, PAMP and DAMP, could trigger further pro-NF-κB signaling throughout the activation of TNFRs and TLRs [53–56]. The deregulated activity of NF-κB is becoming an indicator of most neoplasms processes, including GBM. Evidence emphasizes TLRs induced carcinogenesis connected with NF-κB expression [57,58]. Kina et al. and Ferrandez et al., in studies concerning Glioma, confirmed the participation of the TLR-4/NF-κB pathway in the promotion of carcinogenesis [59,60]. Another interesting relation in glioma genesis is TLR-4/Programmed death receptor-1/Programmed death receptor-1 ligand (PD-1/PD-1L) axis interaction. Overexpression of TLR-4 results in an increase of PD-1L in GBM patients and is associated with unfavorable outcome [61]. Additionally, the increase in TLR-4 expression results in Wnt/claudine signaling forwarding to the progression of GBM and limits the effective apoptosis [62]. Progression of glioblastoma is also controlled and modulated by multiple miRNAs molecules. They control the cell circle of glioma cells on different stages of development. The invasiveness of GBM involves the change in epithelial cells polarity and loss of cell–cell adhesive properties. Down expression of E-cadherins results in modifications in the architecture of glioma cells, loss of integrity, and at last, tumor invasion. Increased expression of miR-10-b, miR-29, miR-146 accelerate invasion of GBM and predict an inauspicious outcome. Overexpression of miR-21 inhibits apoptosis and promotes GBM cell survival. miR 210-3p and miR-93 promote angiogenesis. Moreover, miR 130b, miR 140, and miR 184 are intensively expressed in cases of histological progression of glioma [63–70]. TLR-4 signaling is modulated by multiple miRNAs molecules, and a great number of them inhibit this pathway. Xu et al. challenged the thesis that long noncoding RNA UBE2R2-AS1 targets TLR-4/miR 877-3p and promotes apoptosis and reduces invasiveness in glioma tissue. The study revealed that UBE2R2-AS1 targets miR 877-3p, inhibits its limiting effect on TLR-4, and as a result, promotes TLR-4 dependent apoptosis [71].

3. Potential Immunotherapeutic Targets

Developments made in neuroimmunology and tumor biology revealed interesting properties of TLR. Activation and TLR-4 downsignaling seem to play an interesting role in the promotion of glioma growth and invasion. On the other hand, some studies discovered that, in some special conditions, they could take part in the anticancer response. The release of HMGB1 from GBM dead cells during radio and chemotherapy stimulates the TLR4 pathway. It leads directly to Dendritic Cell maturation and efficient tumor antigen presentation leading to GBM regression in animal models [72–77]. There is adequate evidence clarifying that the modulant of TLR-4 proves efficiency and safety for GBM treatment based on previous trials concerning different neoplasms. The data imply that TLR-4 signaling has a dual disposition as a double-edged sword [61,78]. Taking control over this complex interplay could bring a satisfactory result in GBM matters. miRNAs, immune checkpoints, the Wnt axis, and glioma stem cells (SCS) could be used as a path to the complicated goal of effective glioblastoma therapy.

3.1. miRNA/TLR-4 Interplay

miRNAs represent a group of short noncoding strands of RNA with length oscillating 20–22 nucleotides each. Around 30% of all genes are controlled and regulated by miRNAs. Molecules bind to the targeted mRNA with the 3’ region called the untranslatable (UTR) monitoring the expression of particular genes through degradation or translation breakdown. As a result, miRNAs are engaged in various biological processes, such as homeostasis, growth, differentiation, immune activation, apoptosis, and stress response. Otherwise, they modulate the immune response to pathogen invasion throughout the TLR signaling pathway (modulation of cytokines, transcription factors, and signaling
proteins and receptors). Additionally, miRNA activity is similarly detected in normal healthy tissue in chronic diseases and cancers [79–87].

Levels of miRNAs are significantly higher in GBM tissue and GBM cell lines [87–92]. The impact of those molecules in carcinogenesis is still unclear, requiring further investigation [93]. There is evidence pointing at a strong relation between TLR-4 signaling and miRNA expression. TLR-4/miRNA interplay changes the typical balance between immune response and inhibition. The indirect influence of miRNAs concerning TLR downregulation was represented by the study by Taganov et al. The study team documented lipopolysaccharide-induced expression of miR-132, miR-155, and miR 146a, regulating TLR downregulation [94–96]. Another study connected miR-34a expression with significant antitumor properties in glioma p53 mutant cell line U251. Overexpression of miR-34a induces apoptosis and inhibits the growth of glioma through the activation of numerous particles. A similar effect of miR-34a was revealed by Xu et al. while investigating breast cancer. In their study, the miR-34a molecule was found to inhibit C-X-C motif ligand 10 (CXCL10). The ligand is characterized by a strong affinity to TLR, promoting cancerogenic downregulation [97,98]. Indirect inhibition of TLR signaling results in the regression of tumor growth and expansion. Direct TLR-4 inhibition is induced by different miRNAs [99–102]. miRNA molecules directly develop inhibitory features, in particular during stages of TLR-4 activation. The MyD-88-dependent pathway IRAK1–TRAF6 complex is inhibited by miR 93-5p, miR 302b-5p, miR 124-5p, and miR 146a/b-5p. The TAK-1 complex is inhibited by miR 23b-5p, miR 142-3p, miR 155-5p, and miR 23-5p. Signaling through the Myd88-independent pathway is regulated by miR3178-5p and miR 3473-5p as inhibitors of TRAF-3, and miR 21b-3p, miR 146a-5p, and miR 302c-5p as direct inhibitors of IRF-3 [94,103–108]. TNF-alfa is inhibited by miR 19a-5p, miR125b-5p, and miR 203-5p. IL-6 is blocked by miR 9-5p, miR26a-5p, miR100-5p, and miR 365, and IL-10 is inhibited by miR-98-5p and miR-106a-5p [109,110].

3.2. Immune Checkpoints vs. TLR-4

Immune checkpoints (ICs) are molecules with the ability to modulate T cells. Coinhibitory and costimulatory properties ensure a balanced immune response. ICs protect autoimmunity and optimize the adequate response of the immune system. When carcinogenesis occurs, IC signaling becomes a route of immune escape, leading to aggressive tumor invasion. Cytotoxic T-lymphocyte antigen 4 (CTLA-4) was the first well-described IC molecule expressed on CD-8+ and CD-4+ T cells. CTLA-4 binds to the corresponding B7-1 (CD80) ligand on antigen-presenting cells (APCs). Interaction between both results in the inhibition of T cells. In GBM, CTLA-4 expression on CD8+ and CD4+ has a significant correlation with poor outcome and invasion of brain tissue. TLR signaling promotes the overexpression of CD80, enhancing the presentation of antigens. It strengthens CTLR-4-induced T-cell anergy and GBM expansion [111–114].

The PD-1/PD-1L axis is another IC pathway. PD-1 and PD-1L are the most potent immunomodulatory proteins. PD-1L was described in 1999 by the Dong study team as a B7-H1 molecule [115], which represents the B7 protein family with a homology level of around 20%. The expression of PD-1L could be divided into inducible and constitutive. The constitutive can be met on antigen-presenting cells and resting lymphocytes. Inducible expression appears during inflammation and immune response; PD-1L represents suppressive properties. Glioblastoma cells represent both constitutive and inducible expression of PD-1L [116,117]. The phenomenon of autoinduction through TLR-4 also occurs. TLR-4 activation on GBM results in signaling through the MyD-88-independent pathway, TRAF6/ERK-1/2 AP-1s, leading to the activation of the PD-1L promoter. PD-1L mRNA is translated into ribosomes, modified in the Golgi apparatus, and presented as a mature PD-1L forming on the surface of GBM cells. T cells recognize PD-1L and bind it with an adequate PD-1 receptor. The intracellular domain of the receptor consists of an (ITSM) tyrosine-based switch motif that activates an inhibitory cascade [118–124]. Downsignaling promotes tyrosine phosphatase 2/Zeta-chain-associated protein kinase 7 (SHP-2/Zap 70) interplay, resulting in dephosphorylation and a significant decrease in lymphocytic cytotoxicity and proliferation. Activation of the PD-1/PD-1L axis leads to T-cell anergy and increases the lymphocyte apoptotic ratio.
Lipopolysaccharides (LPSs), high-mobility group box-1 proteins (HMGB1), and the heat-shock-protein (HSP) family act as TLR-4 agonists that initiate signaling promoting PD-1L expression. Most of those molecules are intensively expressed in the GBM microenvironment, resulting in the autocrine induction of regional immunosuppression controlled by the PD-1L/PD-1 axis [125–127].

A study performed by Beswick et al. [128] discussed the matter of TLR-4/PD1L-induced immunotolerance in colon mucosa. Additionally, Wolfe et al. described a similar inductive effect of TLR-4 activation on PD-1L expression [129]. Poor prognosis for patients with peripheral lymphomas, correlated with TLR-4 and PD-1L overexpression, presented by Zhao et al., confirmed the pro-oncogenic effect of the presented axis [130].

3.3. TLR-4/Wnt Axis and Apoptosis

Glioblastoma, as a highly aggressive neoplasm, activates or modulates many pathways promoting gliomagenesis. One of those downsignaling instances concerns wingless signaling (Wnt\ Claudines axis) occurring as a crucial controller of cell–cell interplay, and as a regulator of migration. Any abnormalities in Wnt lead to evolutive effects. Aberrations in Wnt balance between stimulation and inhibition usually promote carcinogenesis. The Wnt complex represents an extended network consisting of various components, crosstalk interactions, and multiple regulatory stages. Wnt usually starts with receptor and coreceptor stimulation. Receptors, such as low-density lipoprotein receptor-related protein 6 (LRP6), protein Tyr kinase 7 receptor (PTK7), Tyr kinase-like orphan receptor (ROR), skeletal muscle Tyr kinase receptor (MUSK), and Tyr kinase receptor (RYK), are treated as Wnt-related. The main regulation of receptors is intracellularly processed by phosphorylating kinases. Extracellular regulation takes place through active agonists, such as norrins, the R-spondin family, and antagonists WNT inhibitory factor (WIF), secreted frizzled-related protein (SFRP), sclerostin, and Dickkopf-related protein 1 (DKK1) [131–137].

Wnt pathway activation is connected with other molecules called claudins. Claudins are a family of proteins that determine tight junctions (TJs) (claudin-5, claudin-3, and claudin-1). Claudin-5 is mainly expressed on the lung epithelium and brain tissue. Claudin-5, built-in TJs, bands in CNS endothelial cells. TJs create direct intercellular adhesion reducing cell-to-cell distance. In effect, they form the real connection between particular endothelial cells, strengthen the integrity of vessels, and control the diffusion of different ions and solutions. CNS embryonal development changes the permeability ratio. An immature form of the CNS vasculature, leaky blood vessels with characteristic fenestrations, are replaced by more integrating TJs. Claudin-5 contributes to the function of the blood barrier [138–142]. Overexpression of claudin-5 reduced inulin diffusion through the brain-vessel endothelium in rat models [143]. On the other hand, the downexpression of claudin-5 promotes epithelial permeability and the loss of blood–brain-barrier (BBB) properties [144]. Lower levels of claudin are correlated with higher levels of TLR-4 expression [145–148].

Dickkopf-related protein-3 (DKK-3), as was mentioned above, represents a group of proteins with strong suppressive features able to halt Wnt signaling. Moreover, DKK-3 seems to be an effective inhibitor of tumor-cell growth molecules. Casili et al. performed a study revealing DKK-3 to have a regulating influence on the Wnt pathway, resulting in increased caspase-3-dependent apoptosis [62].

Caspase-3, a representative of the caspase family, is described as an effector caspase. Its properties, such as activation by many apoptotic inducers, result in rife activity leading to complete apoptosis in many cell lines. Overexpression of caspase-3 results in an apoptotic GBM phenotype, confirmed in patient samples. Studies suggested that a higher apoptotic ratio is associated with longer PFS in treating for GBM. Sensitivity to temozolomide (TMZ) treatment is also correlated with caspase-3 expression, and higher levels of caspase-3 promote TMZ efficiency. Interestingly, caspase-9, a less apoptotic molecule, is downexpressed after Dickkopf WNT Signaling Pathway Inhibitor 3 (DKK-3) upregulation, usually increasing after TLR-4 stimulation. Highly invasive cancer-cell lines express caspase-9 more intensively [62,149–151].
Apoptosis, as a canonical biological interaction, regulates the subsistence of organisms and limits the expansion of GBM. A study connected the expression of DKK-3 and claudine-5 in healthy brain tissue, compared with the downexpression of both in GBM patients. Significantly lower levels of DKK-3 were observed in many types of neoplasms concerning prostate cancer. The absence of TLR-4 inhibits the growth of glioblastoma lines. The expression of TLR-4 interferes with Wnt downsignaling, promoting carcinogenesis. Additionally, the lack of TLR-4 maintains claudin-5 and DKK-3 proapoptotic properties, resulting in the limitation of tumor expansion. To sum up, the downexpression of TLR-4 promotes Wnt/DKK-3/claudin-5 signaling, which limits GBM invasiveness [62,152,153].

3.4. TLR-4 Influence on Non-CSC Glioma Stem Cells

A study performed by Alvarado et al. revealed a lack of TLR-4 occurrence on cancer stem cells (CSCs), the most invasive and most aggressive subpopulations of GBM. In opposition to CSC mature GBM cells and non-CSCs, it demonstrated TLR-4 expression and a response to agonist ligation. CSCs are responsible for therapeutic ineffectiveness and tumor-mass progression. External stimulation, such as necrosis, hypoxia, uncontrolled proliferation, and acidic stress, characteristics of a tumor microenvironment, generate unfavorable conditions. Thanks to developed adaptive mechanisms, CSCs maintain the balance between self-restoration and differentiation in hostile habitats. Owing to limited TLR-4 expression, CSCs can survive in a malevolent environment. Experiments conducted by a study team explained the processes of suppressing the CSC subpopulation, in which TLR-4 signaling played a key role. The main discovery in those papers indicated intensive TLR-4 downsignaling, halting CSC expansion by reducing the activity of retinoblastoma binding protein 5 (RBBP5), which is naturally increased in this subpopulation. Another important thing to understand in the underlying processes in CSCs is that RBBP-5 promotes cardinal stem-cell transcription initiators, essential to achieve invasiveness and self-restoration. TLR-4 expression and downsignaling inhibit RBBP-5 by the phosphorylation of TBK-1. TLR signaling borders proliferative trends in CSC and GBM [154–160].

Recent studies concerning bladder tumors, prostate cancer, and colorectal cancer pointed at TLR signaling as a tumorigenic and tumor-progression factor [161–164]. Dapito et al. linked TLR-4 activation with the significant progression of hepatocellular carcinoma. Alvarado et al. presented the opposite effects, where the receptor limited tumor growth by the direct blockade of the self-restoration cycle. The described divergence of TLR-4 signaling could be linked with ligand properties and downsignaling; TBK-1 seems to be an explanation, which is an element of the TLR-4–MyD88-independent pathway, and activation may also be signaled through the MyD88-dependent pathway, as it occurs in hepatocellular cancer [160,165]. Further observation and experiments explaining these differences are required, which are necessary to understand the anti- and procarcinogenic features of TLR-4. Signaling through the MyD88-independent and MyD88-dependent pathway may bring totally different results. Elucidation of these opposite results is crucial for future perspectives of immunotherapy for GBM (Figure 2).

4. Trials Concerning TLR-4

4.1. In Vitro and In Vivo Agonist

Exposition of TLR-4 on LPS in in vitro studies resulted in tumor-cell-line progression. The promotion and proliferation of the U87 and U118 lines were detected, and an increase in invasion was realized in U87 [28]. Stimulation of LPS increased metalloproteinase-9 (MMP-9), a factor of invasion detected in U87 glioma cell lines. Moreover, parallel TLR-4 signaling modulated the evasion of the immune system, tumor-necrosis-factor resistance, and cell survival [166]. The spirulina complex (polysaccharide) and LPSs as TLR-4 agonists demonstrated antitumor activity in mouse models. Differences in the action of both were underlain in IL-17 interaction. IL-17, with its pleiotropic characteristic, acts as an anti- or protumor cytokine, depending on the investigated neoplasm model. An increase in the spirulina polysaccharide, in contrast to LPS, increased the levels of IL17 in
mouse-model serum. The murine glioma-cell complex becomes more invasive and expands after IL-17 activation [167,168]. Moreover, Fas and TLR-4 contemporary activation results in the disappearance of TLR-4 tumor-promoting properties [169].

**Figure 2.** Potential targets for future immunotherapy concerning TLR-4 signaling in glioblastoma multiforme: Complex of miRNA molecules inhibiting TLR-4 signaling on different stages, programmed death ligand-1 (PD-1L) overexpression stimulated by TLR downsignaling, indirect retinoblastoma binding protein 5 (RBBP5) inhibition by TBK-1 phosphorylation resulting in antineoplastic activity, interplay between TLR-4 and Dickkopf-related protein 3 (DKK-3) inhibition, Wnt pathway signaling restriction, claudin-5 level reduction, and caspase-9 elevation and caspase-3 decrease as a pro-oncogenic cascade after TLR-4 activation.

Prosaposin (PSAP) conserved glycoprotein acts as a neurotropic factor in the glioblastoma environment. High levels of PSAP were detected in glioblastoma patients and were associated with unfavorable outcome [27,170,171]. Jiang et al. investigated PSAP as a promotor of proliferation and tumorigenesis through TLR-4 signaling. Mice models confirmed poor prognosis related to PSAP overexpression. The study hypothesizes PSAP affinity to TLR-4. Co-localization of TLR-4 and PSAP was confirmed with immunofluorescence staining. To sum up, PSAP activates TLR-4/NF-κB signaling, induces secretion of factors responsible for inflammation and tumor growth [172]. The blockade of PSAP becomes an interesting target for improving GBM outcome.

LPS was examined as affecting GSCs, and mature glioma cells in the study performed by Han et al. rat model has proven that LPS stimulation prolonged survival time significantly in the observed glioma-cohort [173]. Interestingly some studies report an antitumoral effect induced by a bacterial infection in animal models and report some cases of GBM patients [174–177]. LPS via TLR-4 stimulation changes immuno-phenotype in GSCs and the mature form of glioma cells and induces antitumoral response [173].

4.2. Agonist and Inhibitors of TLR-4 in Oncology

TLR-4 is expressed on the surface of the cell membrane and occurs on endosomes. Moreover, the receptor has the ability to dependently signal to MyD88 and TRIF complexes. Those characteristic features are the example of evolutionary controlled carefulness in precise antigen-detection
and downsignaling-promotion mechanisms. TLR-4 coreceptor myeloid differentiation factor 2 (MD-2) has an appropriate pocket that binds the corresponding ligands. Immunotherapy disrupting TLR-4 and MD-2 binding or blocking their interplay was explored [178–180]. Another interesting ligand inhibiting or activating TLR-4, containing a glucopyranosyl lipid adjuvant (GLA) agonist [181,182], monophosphoryl lipid A (MPLA) agonist [183,184], and lipid 4A antagonist, was discovered and explored [185]. Interestingly, TLR-4 is the most popular among the TLRs evaluated as receptors in numerous clinical trials as a potential target for immunotherapy against various pathologies concerning inflammation, viral infection, immune diseases, and cancers (Table 3) [186–188].

Table 3. First- and second-phase clinical trials concerning TLR-4 agonists and antagonists in the treatment of various neoplasms.

| Clinical Trial Number | Phase | Indication             | Agonist/Antagonist of TLR-4 | Ligand Characteristic |
|-----------------------|-------|------------------------|-----------------------------|----------------------|
| NCT 02320305          | 1     | Melanoma               | Agonist                     | MART-1               |
| NCT 02180698          | 1     | Soft tissue sarcoma    | Agonist                     | GLA-SE               |
| NCT02501473           | 1,2   | Follicular lymphoma    | Agonist                     | G100                 |
| NCT02995655           | 1     | Acute myeloid leukemia | Antagonist                  | CX100                |
| NCT02035657           | 1     | Merkel cell carcinoma  | Agonist                     | GLA-SE               |
| NCT02270372           | 1     | Breast and ovarian cancer | Agonist                   | ONT-10               |
| NCT01556789           | 1     | Solid tumors           | Agonist                     | ONT-10               |
| NCT02609984           | 2     | Sarcoma                | Agonist                     | CMB-305              |
| NCT02387125           | 2     | Non-small lung cancer  | Agonist                     | CMB-305              |

4.3. TLR-4 in Glioblastoma Multiforme—Clinical Application

The macrophage migration inhibitory factor (MIF) cytokine is treated as a potent cytokine initiating immunity and inflammation processes [189–191]. MIF is excreted by macrophages immediately after the ligation of bacterial products and inflammatory molecules. Studies showed a strict interplay between MIF and TLR-4 signaling. MIF knockout mice presented the downregulation of TLR-4 signaling. The response of macrophages to LPS stimulation was significantly limited [192–195]. The presented model made the basis for immunotherapy targeting MIF and TLR-4. Trial NCT03782415 contested ibudilast MN-166 in Phase 1 and compared it with TMZ combo treatment in recurrent GBM in Phase 2. A multicenter open-label study evaluated its efficacy, tolerability, and safety with a TMZ combination in 50 recurrent GBM patients. To be eligible, patients required a Karnofsky Performance Scale (KPS) > 70 (https://clinicaltrials.gov/ct2/show/NCT03782415).

Ibudilast is an agent that is able to diffuse the blood–brain barrier (BBB), developing antitumor activity by inhibiting MIF and phosphodiesterase-4 (PDE-4), also reducing TLR-4 downsignaling with pro-oncogenic properties. Action leads to excessive apoptosis and the limitation of cell proliferation in GBM [196–198]. Ibudilast was also observed in trials, including neuroinflammation, amyotrophic lateral sclerosis (ALS), dysphoria, treatment of alcohol-use disorders, and methamphetamine dependence (https://clinicaltrials.gov/ct2/results?cond=ibudilast&term=&cntry=&state=&city=&dist=).

Another clinical application of TLR-4 signaling concerns the antiglioma HSP vaccination. HSP, as a part of the heat shock protein peptide complex (HSSPC) (Table 4), is characterized by a strong affinity to numerous receptors on the surface of the cell. T-cell supported HSP stimulation of APCs results in downstream signaling and NF-κB activation. CD91/CD40/CD36/CD14/TLR-2 and TLR-4 seem to play crucial roles in this process. Promotion of the TLR-4/NF-κB axis in APC causes intensive secretion of chemokines and proinflammatory molecules to the tumor microenvironment. Ipso facto, HSP stimulates, indirectly, the release of inflammatory factors, such as IL-12, IL-1beta, TNF alfa, granulocyte macrophage colony-stimulating factor (GM-CSF) and inhibits GBM growth [199–206].
Table 4. Clinical trials concerning heat shock protein peptide complex (HSSPC) vaccination in glioblastoma-multiforme (GBM) patients. (https://www.clinicaltrials.gov).

| Clinical Trial Number | Phase | Immunological Agent | Number of Participants | Indication | Age | Country |
|-----------------------|-------|---------------------|------------------------|------------|-----|---------|
| NCT03650257           | 2     | Autologous Heat Shock Protein (gp96) Vaccine | 150 | GBM | Adult | China |
| NCT02722512           | 2     | HSPPC-96 Vaccine | 20 | hGG, rGBM, GBM, Ependymoma | Child/Adult | USA |
| NCT03018288           | 22    | Pembrolizumab (anti PD-1), HSPPC-96 Vaccine | 108 | GBM | Adult | USA |
| NCT00905060           | 2     | HSPPC-96 Vaccine | 46 | GBM | Adult | USA |
| NCT00293423           | 1/2   | HSPPC-96 Vaccine | 41 | rGBM | Adult | USA |
| NCT01814813           | 2     | HSPPC-96 Vaccine with Bevacizumab | 90 | rGBM | Adult | USA |

The study performed by Crane et al. [207] reported significant immunization after HSP administration in 11 out of 12 patients with recurrent GBM (rGBM). Median Survival time in the group of immune responders oscillated about 47 weeks after surgical resection and vaccination. Non-responding patients survived 16 weeks only. Promising results encouraged for further investigation. Bloch et al. revealed a median overall survival oscillating 42 weeks (42.6 weeks) after HSPPC-96 administration in rGBM patients. Further efforts concerning the design of Phase 2 studies have been performed [208]. Clinical trials challenging the effectiveness and safety of HSPPC-96 vaccination evaluate overall survival and progression-free time. Trials also compare results with standard treatment, other immunological agents (Bevacizumab, Pembrolizumab), and the combination of drugs (https://www.clinicaltrials.gov) (Table 4).

5. Conclusions

TLR-4 signaling pathways and their wide spectrum of interactions seem to be promising targets for immunotherapy. Multimodal interplay and a variety of downsignaling encourage TLR-4 to be further considered as a potential aim for immune agents. Clinical trials contesting TLR-4 agonists and inhibitors are recruiting patients with various cancers. TLR-4-related treatment is being gradually introduced for GBM patients. There is still much to do in the field of effective immunotherapy.

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