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Review

Taking a Toll on Self-Renewal: TLR-Mediated Innate Immune Signaling in Stem Cells

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Innate immunity has evolved as the front-line cellular defense mechanism to acutely sense and decisively respond to microenvironmental alterations. The Toll-like receptor (TLR) family activates signaling pathways in response to stimuli and is well-characterized in both resident and infiltrating immune cells during neural inflammation, injury, and degeneration. Innate immune signaling has also been observed in neural cells during development and disease, including in the stem and progenitor cells that build the brain and are responsible for its homeostasis. Recently, the activation of developmental programs in malignant brain tumors has emerged as a driver for growth via cancer stem cells. In this review we discuss how innate immune signaling interfaces with stem cell maintenance in the normal and neoplastic brain.

Trends

TLRs are expressed in a variety of non-immune cells.

TLRs have non-immune functions in neural cells during development and in the adult brain.

TLR expression has not been extensively studies in the glioma tumor microenvironment.

TLR stimulation can have both pro- and anti-tumorigenic activities depending on the downstream effectors in the cell expressing the receptor.

TLR Origins and Biology

The Toll receptor was first described in *Drosophila*, where it is essential for the establishment of dorsoventral patterning during development [1]. Toll is similarly involved in the control of antifungal responses in the adult fly [2]. The first Toll receptor homolog was later identified in humans [3], and eventually a family of Toll-like receptor (TLR) proteins was described, the major function of which is to mediate recognition of both pathogen and damage-associated molecules. The TLR family now consists of 10 members in humans (TLR1–TLR10). The cytoplasmic region of all TLRs is very similar to that of the interleukin (IL)-1 receptor family, and is known as the Toll/IL-1 receptor (TIR) domain [4]. Despite this common feature, TLRs are capable of recognizing different molecular patterns to elicit a response. While the role of TLRs has been well characterized in the innate immune system, several additional functions have not been completely understood or explored, such as their function during development, which was the original observed role of the *Drosophila* Toll receptor.

In this review we examine recent evidence highlighting the importance of TLRs in development in both physiological and malignant backgrounds. First, we introduce the known roles of TLRs in the brain in a physiological context, in both embryonic and adult stem cells. This will then be contrasted with recent discoveries made in several types of cancer and, more specifically, in brain tumors. Finally we discuss how the identification of downstream molecules mediating the differential function of TLR signaling in normal and neoplastic cells will be crucial for the design of future therapeutic strategies.

TLR Expression and Function in Neural Development and Disease

Although the role of TLRs has been well studied in immune processes, TLR expression is not exclusively limited to immune cells (i.e., macrophages, dendritic cells, neutrophils, T cells, and B cells) but has also been reported in multiple other cell types under physiological conditions, including nervous [5–7], muscular [8,9], reproductive [10,11], colonic [12], adipose [13,14], renal

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Box 1. Innate Immunity and Inflammation

Pathogen-Associated Molecular Patterns (PAMPs)

PAMPs are derived from microorganisms and recognized by pattern recognition receptors (PRRs, which include TLRs) expressed in cells of the immune system [84]. During a microbial infection, PAMPs alert the immune system of the presence of pathogens, which will induce innate immunity [85] and subsequent inflammation and cellular processes including phagocytosis, complement, and cytotoxicity via natural killer cells.

Damage-Associated Molecular Patterns (DAMPs)

DAMPs are endogenously derived molecules released by either damaged cells following injury or from the extracellular matrix upon tissue damage; these molecules can also be recognized by PRRs (a complete list of endogenous ligands and the TLRs they activate can be found in [20]).

TLR Activation Following PAMP and DAMP Recognition

Although both types of molecules can be recognized by the same type of receptors, there are some differences in ligand recognition and TLR activation. In the case of DAMPs, TLRs become activated by dimerization of the receptor (mainly by homodimerization, but TLR2 can also form heterodimers with TLR1 and TLR6) to achieve specific ligand recognition [86]. By contrast, recent evidence suggests that DAMPs depend more on coreceptors and accessory molecules [87]. For example, when TLR4 and CD14 are present in the membrane a set of ligands can be recognized (lactoferrin, surfactant proteins A and D) whereas if TLR4, CD14, and MD-2 are present then a different set can be recognized (HSP60 and 70, oxidized LDL, S100A8, S100A9, biglycan); reviewed in [20]).

Infectious and Sterile Inflammation

Mounting an inflammatory response is the first crucial step in innate immunity to combat the presence of a dangerous threat (either a pathogen or endogenous ligands). The mechanisms elicited by the innate system are potent and effective in killing pathogens, but can also damage host cells [88]. This collateral damage is minimized by immune clearing mechanisms that dampen the activity of recruited effector leukocytes. Conversely, DAMPs can activate inflammatory cascades in a process termed sterile inflammation. The presence of endogenous ligands induces the recruitment of innate immune cells that will mainly cause tissue damage and, if unresolved, could exacerbate the immune response and promote a chronic condition.

[15,16], hepatic [17], and alveolar [18] tissue. A complete tissue-specific mRNA expression profile of human TLRs can be found in [19]. This diverse expression is not surprising based on the description of TLRs as receptors for not only pathogen-associated molecular patterns (PAMPs) but also endogenous damage-associated molecular pattern (DAMP) ligands. DAMPs are key danger signals that are released in response to tissue damage that initiate a repair process [20] (see Box 1 for a detailed description of these molecules). However, DAMPs can also play a role in the progression of pathogenesis by stimulating inflammation and releasing cytokines. This is true in both autoimmune diseases such as rheumatoid arthritis [21], multiple sclerosis [22], and cancer, where necrosis is a hallmark of malignant progression [23,24]. Perhaps the most remarkable feature of this function of TLRs is the ability of the same receptor to recognize, despite their diversity, several endogenous ligands (proteins, fatty acids, or degradation products of the extracellular matrix, listed here [20]). This promiscuity can be explained by the fact that TLRs can exist in the membrane in the presence of several different co-adaptor molecules that are able to modulate the interaction with various ligands, thereby providing specificity. Therefore, the capacity of cells to respond to several stimuli in a physiological setting can be transposed to the involvement of TLRs in several malignancies. For example, there has been an emphasis on studying TLR4 signaling based on its association with several diseases, including neurodegeneration, traumatic brain injury, Alzheimer's disease, multiple sclerosis, Parkinson's disease, and amyotrophic lateral sclerosis [25].

In the brain, an organ that has unique and specialized immune response mechanisms, TLRs have been described in resident, non-immune neural cell types including microglia, astrocytes, oligodendrocytes, neurons, and neural progenitor cells (NPCs) [5–7]. Both microglia and astrocytes express virtually all known TLRs, while oligodendrocytes, neurons, and NPCs

Table 1. TLR Expression in Neural Cells

	Microglia	Astrocytes	Oligodendrocytes	Neurons	Neural Progenitor Cells
TLR1	✓	✓		✓	
TLR2	✓	✓	✓	✓	✓
TLR3	✓	✓	✓	✓	✓
TLR4	✓	✓	✓	✓	✓
TLR5	✓	✓			
TLR6	✓	✓			
TLR7	✓	✓			
TLR8	✓			✓	✓
TLR9	✓	✓			
TLR10		✓			

express TLR2, TLR3, and TLR4 (Table 1). Of note is the expression of TLR8 in both neurons and NPCs. The differential expression of TLRs in distinct cellular populations is interesting and deserves detailed exploration, given that microglia are the only resident cell type thought to serve immune-related functions in the brain [26]. In addition, while the presence of a particular receptor subtype is capable of mediating the response to a specific ligand, the selective recruitment of adaptor proteins and downstream effector activation contribute substantially to the receptor-mediated response to particular ligands. As reviewed in [27], TLR4 signaling in neural cells is different from canonical dendritic cell TLR4 signaling [28], in which stimulation by lipopolysaccharide (LPS), a TLR4 ligand, leads to activation and translocation of nuclear factor κ light-chain-enhancer of activated B cells (NF-κB) [in a myeloid differentiation primary response gene 88 (MyD88)-dependent manner].This ultimately results in increased expression of tumor necrosis factor-α (TNF-α), IL-6, and IL-12. TLR4 signaling is similarly capable of inducing interferon (IFN) regulatory factor (IRF)-3 activation (which, in contrast to the above, is MyD88-independent). Microglia and astrocytes exhibit comparable TLR4 signaling in response to LPS, but astrocytes are unable to activate IRF-3 [29]. Moreover, TLR4 activation in neurons does not appear to induce the canonical downstream signaling, and different adaptor molecules are present in the membrane (i.e., MD1 instead of MD2 as in all other cell types) [30]. Similar differential responses were observed when human microglia and astrocytes were stimulated with synthetic lipopeptide (PAM), synthetic dsRNA polyinosinic:polycytidylic acid [poly(I:C)], or *E. coli* LPS [7]. In the case of microglia, both poly(I:C) and LPS were able to induce significant levels of TNF-α, while all three stimuli were capable of eliciting IL-6 release. By contrast, astrocytes were only responsive to stimulation of TLR3 with poly(I:C), which led to the robust secretion of IL-6 but no detectable levels of TNF-α. These observations further underscore the importance of downstream effector selection in the presence of various stimuli under physiological conditions.

In the context of NPCs and neural development, there is evidence that TLRs are involved in cellular proliferation [25,31–33], differentiation [33–35], and survival/migration [34,36,37] at distinct developmental stages. For example, deficiency of TLR3 increases proliferation of subventricular zone-derived embryonic NPCs [31]. Similarly, treatment of these same cells with activating ligands LPS (for TLR4), poly(I:C) (for TLR3), and PAM (for TLR2) caused a decrease in proliferation [31,33]. In the adult mammalian brain, deficiency of TLR4, MyD88, or TRIF increases the proliferation of dentate gyrus-derived NPCs. This effect is not recapitulated by either TLR2 or TLR3 deficiency [27]. In addition, stimulation of TLRs with LPS (for TLR4) or poly(I:C) (for TLR3) inhibits adult NPC proliferation [32,33,38]. Finally, more recently, it was shown that TLR9 stimulation with CpG oligodeoxynucleotide (ODN) in NPCs resulted in the release of neuroprotective molecules such as the chemokine receptor CX3CR1 and triggering receptor

expressed on myeloid cells 2 (TREM2). This in turn switched microglia from a proinflammatory to an anti-inflammatory, protective phenotype [39]. Although some of the endogenous ligands that bind TLRs to activate downstream effectors have been described, there is still room for exploration in this regard. Nevertheless, the expression of TLRs in distinct adult populations clearly emphasizes their role in neural cells to achieve normal homeostasis and proper function.

TLRs in the Oncogenic Context

A link between development and disease has been well established: for example, tumor progression is often dependent on the utilization of key developmental pathways. Numerous TLRs have been associated with tumor development and progression in several malignancies, including breast [18,40,41], colon [42–44], pancreas [45,46], prostate [47,48], liver [49], lung [50,51], leukemia [52–54], and ovarian [55,56] cancers, among others. In general, independent of the specific TLR protein, activation of these receptors is linked to the recruitment of immune cells and the release of proinflammatory cytokines. This in turn has been associated with a favorable environment for tumorigenic cells to thrive and disproportionately proliferate.

Although expression of most TLRs has been shown for the above-mentioned tumors (using immunohistochemistry and RNA screening techniques), the expression of TLRs is not necessarily associated with an oncogenic setting. This family of receptors has also been studied in different situations. For example, in breast cancer, expression of all TLRs has been examined [18,40], but to date only TLR4 expression levels have been targeted. Decreased TLR4 levels inhibited proliferation and survival of breast cancer cells *in vitro* [18]. In another model, TLR7 and TLR8 expression was examined, together with that of CD133, a canonical marker of self-renewing cancer stem cells (CSCs). These two TLRs were found to also serve as CSC markers in colorectal cancer [42]. TLR1, TLR2, TLR4, and TLR8 expression levels were also found to be higher, together with downstream targets such as IL-6, in colorectal cancer patient tissue compared with normal mucosa [44].

It is of paramount importance to carefully study where these TLRs are expressed and the downstream cascades that are activated so as to increase our understanding of TLR function and develop efficient therapeutics. In the following sections we will focus on TLRs in brain tumors: specifically, in individual cell populations where TLRs are involved in a myriad of malignant processes, such as invasion, migration, proliferation, and immunosuppression. Knowledge of the function of TLRs in normal brain development and function may be leveraged to identify key regulatory mechanisms for tumor growth and progression that could ultimately be therapeutic targets.

Glioblastoma (GBM): A Therapeutic Challenge

GBM is the most common malignant primary brain tumor in adults. Despite recent advances in treatment, this disease remains uniformly lethal. The observation of cellular heterogeneity within GBM led to the identification of a subset of self-renewing CSCs [57–60]. This population is characterized by resistance to chemotherapy [61] and radiotherapy [62], and is currently the target of a new generation of antitumor drugs [63]. In addition to malignant cells, the tumor microenvironment is also composed of tumor-infiltrative cells and endothelial cells that, together with the physical location of the tumor, contribute to regulating the function of CSCs. Thus, GBM CSCs can be regulated by a variety of both intrinsic and extrinsic mechanisms. Intrinsic factors include genetics, epigenetics, and metabolism, while extrinsic factors refer to niche factors, the cellular microenvironment, and the host immune system [64]. GBM has been a prototypic tumor for the development and testing of novel targeted therapies (including specific pathway inhibitors and agents targeting key biological processes such as angiogenesis [65,66]). Despite promising preclinical and early-stage clinical trial success, these approaches have translated to limited therapeutic efficacy. Recently, there have been promising advances in immunotherapeutic

approaches for glioblastoma treatment, many of which have been inspired by the success in other tumor types such as melanoma [67]. These approaches encompass cellular (adoptive transfer or chimeric antigen receptor T cells and bispecific T cell engagers), vaccination [tumor-specific antigens such as epidermal growth factor receptor (EGFR) vIII and tumor-associated antigens such as ephrin type-A receptor 2 (EphA2) or IL-13 receptor subunit α2 (IL13Ra2)], and immunomodulatory [programmed death 1 (PD1), PD1 ligand 1 (PD-L1), and cytotoxic T lymphocyte-associated protein 4 (CTLA4) blockade] techniques [68].

TLRs in Gliomas: Expression in Different Cell Types

Stimulation of the innate immune system with the goal to boost the eradication of tumor cells is part of the novel efforts of glioma immunotherapy. Based on their role in the immune response, TLRs are likely candidates for adjuvant therapies. Indeed, several clinical trials at various stages are using TLR ligands for this purpose (most are still in the patient recruitment stage; summarized in [69]). However, as in the normal brain where non-immune cells utilize TLRs for a variety of functions, the expression of TLRs on tumor cells has not been exhaustively explored, and TLR stimulation can lead to pro-tumor effects if caution is not applied.

In this context, TLR9 has been found to be elevated in GBM CSCs [70]. In patients, high TLR9 expression is associated with poorer survival compared with low expression in several cancers, including GBM [71,72]. When CSCs were treated with the TLR9 ligand CpG-ODN, an induction in Janus kinase 2 (JAK2) activation was observed in a Frizzled 4-dependent manner [70]. This is turn could lead to the induction of signal transducer and activator of transcription 3 (STAT3), a known key transcription factor for CSC maintenance [73–75]. While targeting this interaction using siRNA *in vivo* using an immune-deficient mouse model [70] has been shown to significantly increase tumor latency, several factors must be considered before moving intro clinical trials. That is, we need to consider the impact of targeting TLR9 globally in all cell populations present in the brain that could have a detrimental effect on normal brain homeostasis. For example, as described earlier, TLR9 stimulation in NPCs leads to the release of neuroprotective chemokines and promotes a more anti-inflammatory microglia phenotype [39]. Thus, we need to discover downstream molecular targets that will not alter brain homeostasis.

TLR4 expression has also been reported in glioma cell lines [76–78]. When TLR4 signaling was disrupted in glioma cells treated with TNF-α there was a decrease in the induction of the transcription factors IRF3 and STAT1 together with IFN-β and inflammatory cytokines [78]. In addition, treatment of cells with IL1-β induced the expression of the non-classical human leukocyte antigen (HLA) class I antigen HLA-G and TLR4 in a hypoxia-inducible factor (HIF)-1α-dependent manner. This is relevant to CSC biology because HIFs have been reported to be associated with CSC maintenance and tumor progression [79,80]. Interestingly, β-defensin 3, the expression of which has been found to be elevated in GBM specimens, prevented the signaling of this pathway and the release of proinflammatory mediators [76]. Moreover, treatment with either Fas ligands or LPS led to increased cell proliferation; however, when both ligands were combined, there was an anti-proliferative effect with a concomitant decrease in cell migration and matrix metalloprotease (MMP)-9 expression [77]. These data demonstrate the complex role of TLR family members in GBM.

In the tumor microenvironment, which is enriched in resident and infiltrating immune cells, glioma cells are not the only cells that express TLRs. Thus, the regulation of tumor progression and CSC maintenance could be indirectly driven by TLR signaling in these immune cell populations. Certainly, resident microglia and macrophages present in the tumor niche are capable of interacting with CSCs to regulate their tumorigenic potential. For example, stimulation of TLR2 on the surface of microglia induced membrane type 1 (MT1) MMP expression [81]. This will have pro-tumorigenic effects because MMPs are critical for extracellular matrix degradation

and promote the proliferation and invasion of malignant tumor cells. Likewise, intracranial injection of GL261 cells in a TLR2 knockout background yielded smaller tumors. In a similar study [82], it was demonstrated that glioma supernatant (obtained from the media of cells cultured *in vitro*) upregulates the expression of TLR2 in microglia with concomitant expression of MMP9; these effects can be suppressed by the antibiotic minocycline (other than blocking protein translation, tetracyclines have also been found to inhibit MMPs) and prolong survival in glioma-bearing mice. This further underscores the importance of MMPs for the degradation of the extracellular matrix during glioma invasion, as previously reported [83]. It would be interesting to examine if the roles of TLRs during development and adult neurogenesis, in microglia, astrocytes, and NPCs, are associated with GBM tumor initiation and progression. Although limited, the study of cells that express TLRs in the glioblastoma tumor microenvironment has shed light on new interactions that could be explored for future therapeutics.

Concluding Remarks and Future Perspectives

The evidence presented thus far delineates important roles played by TLRs in microglia, astrocytes, neurons, NPCs, and tumor cells in both physiological and malignant settings discussed in this review (Figure 1). Although the role of TLRs in normal homeostasis in the adult brain is well defined, their involvement has not been carefully characterized in the context of brain tumors, which share hallmarks of development, including a self-renewing stem cell population. While several reports indicate that TLRs are expressed in glioma patient tissue, the cell types that express TLRs in a heterogenic tumor such as GBM, and the functional role of TLRs in this setting, remain open questions that need to be addressed. For example, in which situations would it be advantageous for a cell to induce or suppress expression of a TLR? One can envisage a scenario in which expression of TLRs in cancer cells allows the cells to respond to the stimulus by releasing proinflammatory cytokines that in turn govern the behavior of endothelial cells, microglia, and infiltrating immune cells. Conversely, expression of TLRs can also be detrimental, because DAMPs present in the tumor microenvironment serve as ligands for these receptors. Necrosis, a hallmark of tumor progression, induces the release of several DAMPs that can activate downstream pathways that inhibit proliferation, self-renewal, and invasion. Numerous reports provide evidence for the former point of view; however, if we consider the fact that TLR3 and TLR4 stimulation can act as a negative regulator of proliferation in NPCs [31,32], then we can state that TLRs can be associated with non-immune roles when different signaling partners are involved. Similar questions need to be explored to obtain further insight into the regulation of TLRs and their association with the tumor microenvironment (see Outstanding Questions).

Determining the contribution of each cell population to the establishment of tumors could be achieved by labeling and lineage-tracing techniques. However, experimental challenges are anticipated based on our incipient knowledge of glioblastoma biology. Moreover, the emergence of CSC interactions with the tumor microenvironment as a therapeutic target provides an additional layer of complexity that needs to be accounted for. Elucidating whether TLRs are involved in this communication will contribute to the development of new therapies. Current immunotherapies target blockades in the immune system that suppress its function. If limited success is seen, a combinatorial approach with TLR modulation could benefit patients by eradicating malignant cells. For this approach we will first need to elucidate key molecules downstream of TLRs that are aberrantly activated in cancer cells to design therapies that will not dampen the function of normal, anti-tumorigenic immune cells.

Similarly, more refined experimental approaches will be necessary to account for the complexity of GBM and other advanced tumors. Current preclinical glioma studies are based on the use of cell lines that were established decades ago, lacking cellular heterogeneity, or patient-derived xenografts, the latter being the better option. Nevertheless, in both cases the use of immune-compromised mice is necessary for tumor initiation and growth. This clearly hinders the ability of

Outstanding Questions

Are all TLRs elevated in CSCs or are a subset reduced due to anti-proliferative functions similar to those observed in NPCs?

In what context will TLR activation be important for tumor growth?

Could targeting TLRs be an antitumor therapy?

What role do TLR coreceptors play in determining the specificity of pro- or anti-tumorigenic role?

What is the individual contribution to tumor growth when tumor cells and infiltrating immune cells are simultaneously activated with TLR agonists?

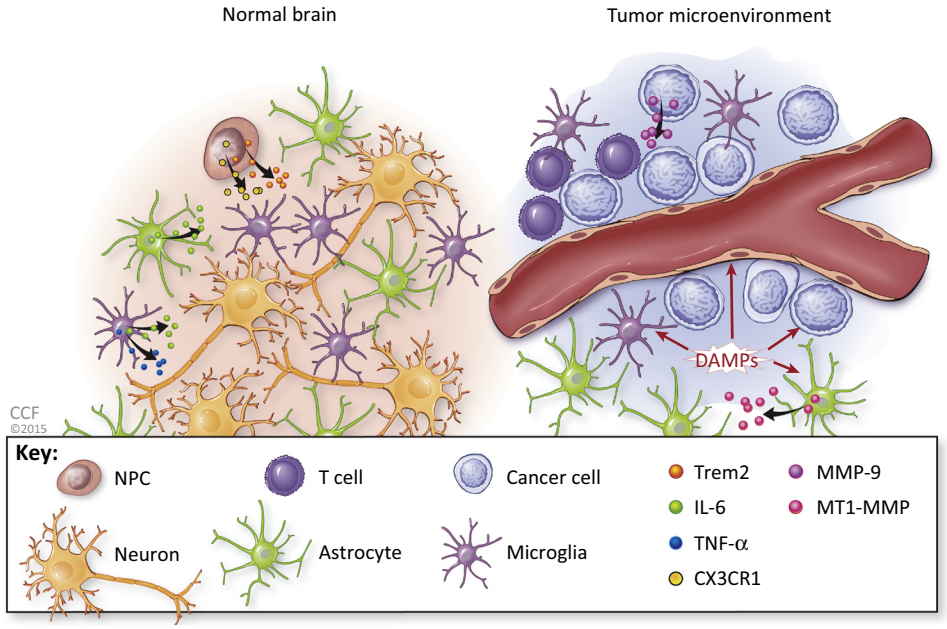

Figure 1. TLRs in the Normal Brain and Glioma Tumor Microenvironment. (A) In the normal brain, stimulation of neural progenitor cells (NPCs) with both lipopolysaccharide (LPS; TLR4 ligand) and polyinosinic:polycytidylic acid [poly(I:C); a TLR3 ligand] leads to a decrease in proliferation [32,33,38] while stimulation with CpG oligodeoxynucleotides (CpG; TLR9 ligand) induces secretion of neuroprotective factors CX3CR1 and TREM2 [39]. For microglia, stimulation with LPS, poly(I:C), and synthetic triacylated lipopeptide (PAM; TLR2 ligand) induces the secretion of interleukin (IL)-6 but only LPS and poly(I:C) promote the secretion of tumor necrosis factor (TNF)-α. Similarly, astrocytes only respond to poly(I:C) and secrete IL-6 but not TNF-α [7]. (B) By contrast, LPS treatment of cancer cells promotes proliferation and induces the secretion of matrix metalloprotease (MMP)-9 [77]. TLR9 has also been found to be elevated in cancer cells [71,72]. Stimulation of TLR2 with PAM leads to secretion in MT1-MMP [81], which is crucial for extracellular matrix degradation in glioblastoma [83]. The presence of damage-associated molecular patterns (DAMPs; depicted in light blue) and which cells can be stimulated by these requires further investigation. This panel also shows the presence of blood vessels (red) in the tumor microenvironment.

these models to incorporate an immune system component, and therefore they are ill suited to analyze the complexity of the interaction of cancer and tumor cells via TLR signaling. It is crucial to asses TLR modulation in cancer cells in the presence of immune cells that will also respond to the same stimuli. For a more comprehensive understanding, mouse models with an intact immune system are needed, and thus syngeneic models would be appropriate; however, these are only available for some tumor types.

In summary, there is an essential role for TLRs in neural development and plasticity, as well as being involved in several key malignant processes in glioblastoma. Future research should aim to move beyond the idea that TLRs only promote inflammatory pathways and the recruitment of immune cells, and instead see these proteins as mediators of a variety of cellular processes that can be pro- or anti-tumorigenic in particular spatial and temporal contexts.

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