DISCUSSION
IGF signaling plays an important role in malignant transformation and protection from apoptosis in a broad range of human cancer [211, 307]. Glioma produces IGFs and express elevated levels of IGF receptors compared to normal brain tissue [308, 309]. Besides, inhibition of IGF receptor disrupts a pro-survival and pro-angiogenic IGF-HIF pathway in glioblastoma cells [283]. Our finding suggests IGF-1 induces HIF-1α activation in glioma cells. We have reported that HIF-1α activation in IL-1β treated glioma cells is dependent on NFκB [216]. However, IGF-1 had no effect on NFκB activation in these cells.

Increase in HIF-1α was concomitant with increase Ras activity and CaMKII phosphorylation. Inhibition of Ras activity through expression of DN-RasN17 abrogated IGF-1 induced increase in HIF-1α activity, establishing Ras as an upstream regulator of HIF-1α activation. Increased Ras expression was accompanied by increased phosphorylation of Ras effectors Akt and ERK in IGF-1 treated glioma cells. Akt activation augments HIF-1α expression in glioma cells [263] and ERK signaling regulates HIF-1α activation [214]. Although selective inhibition of Akt and ERK individually abrogated IGF-1 induced HIF-1α expression, co-treatment with Akt and ERK inhibitors resulted in greater decrease in HIF-1α activity in IGF-1 treated cells. Akt activates mTOR which is deregulated in glioblastoma [227] and mTOR phosphorylates p70 ribosomal S6 kinase (p70S6 kinase) that regulates translation of proteins involved in cellular proliferation and formation. An increase in mTOR and p70S6 levels concurrent with elevated pAkt level was observed in IGF-1 treated cells.

PI3K-Akt signaling pathway also regulates the expression of FOXO- a transcription factor involved in cell survival [264]. Akt activation induces FOXO-1 phosphorylation and nuclear exclusion [265] and phosphorylation mediated inactivation of FOXO increases HIF-1α transcriptional activity [266]. However, IGF-1 had no effect on FOXO-1 phosphorylation or its accumulation in the cytoplasm or nucleus. Neither did treatment with IGF-1 have any effect on FOXO-1 transcriptional
activation. GSK3β is known to mediate HIF-1α destabilization [267] and pGSK3βSer9 is essential for the stabilization of β-catenin [268]. Although β-catenin interacts with HIF-1α to promote cellular adaptation to hypoxia [269], treatment with IGF-1 had no effect on β-catenin expression.

In addition to Ras, IGF-1 induced CaMKII also regulates HIF-1α activation in glioma cells. However, CaMKII mediated HIF-1α activation occurs independently of Ras, as elevated CaMKII levels are unaffected in cells transfected with DN-RasN17. IGF-1 induced increase in HIF-1α activation is concomitant with decreased TLR9 and CXCR4 levels and elevated SOCS3 expression. The most important finding of this study was the identification of a complex crosstalk between TLR9 and HIF-1α in response to IGF-1, under normoxia. TLR9 and HIF-1α seem to regulate each other through two opposite feedback loops. While knockdown of HIF-1α decreases TLR9 by behaving as mutual regulator of each other in a positive regulatory loop, activation of TLR9 signaling abrogates HIF-1α activation through a negative regulatory loop. This finding highlights the complexity of TLR9 signaling in HIF-1α activation and vice versa in glioma cell, as both negative and positive TLR9-HIF-1α feedback loops act in tandem to regulate inflammatory response. It is possible that the negative feedback occurs beneath a given threshold of the two proteins and that the positive loop starts when this threshold is crossed.

As TLR9 signaling regulates both CaMKII and HIF-1α in IGF-1 treated cells and since CaMKII triggered by TLR ligands promote inflammatory responses [271], it is likely that TLR9 act as a sensor to maintain the inflammatory millieu through regulation of CaMKII and HIF-1α. We have reported that HIF-1α maintains persistently high level of IL-1β through an HIF-1α-IL-1β autocrine loop in glioma cells [216]. Pro-inflammatory cytokines in the tumor microenvironment plays a major factor in tumorigenesis [310]. The simultaneous increase in SOCS3 and decrease in TLR9 was concurrent with heightened level of IL-1β and pro-inflammatory cytokines. IGF-1 also elevated the expression of immuno-modulatory cytokine IFNβ.
It is known that TLR9 promotes inflammatory response [271] and SOCS3 prevents IL-1β mediated cytotoxicity [287]. As TLR9 activation decreases IGF-1 mediated increase in SOCS3 expression, it is tempting to speculate that low TLR9 levels maintains elevated SOCS3 expression in IGF-1 treated cells to prevent pro-inflammatory cytokines from crossing the threshold beyond levels required to sustain a TLR9-HIF-1α axis. It is possible that negative TLR9-HIF-1α axis is initiated to maintain elevated HIF-1α levels in presence of IGF-1. Another important finding of this study is the elucidation of the involvement of HIF-1α in the regulation of SOCS3. Over-expression of SOCS3 reduces STAT3 phosphorylation in breast cancer cells [311]. SOCS3 inhibits IGF mediated STAT3 activation [312], and STAT inhibition reduces LPS-induced SOCS-3 expression [132]. Importantly, STAT3 and STAT1 negatively regulate each other through the induction of SOCS [244] and STAT1 functions as a negative regulator of HIF-1α-dependent transcription [281]. IGF mediated increase in STAT1 and decrease in STAT3 phosphorylation was concurrent with elevated SOCS3 expression in glioma cells.

While CXCR4 modulates TLR9 mediated signaling [237], TLR9 increases metastatic potential of cancer cells through CXCR4 expression [236]. Although TLR9 agonist has no contribution towards regulating the elevated pro-inflammatory cytokines levels triggered by IGF-1, treatment with TLR9 agonist alone elevated pro-inflammatory cytokines to levels similar to IGF-1 treated cells. TLR9 agonists stimulate innate and adaptive anti-tumor immune responses and have demonstrated potential for the treatment of cancer [313, 314]. This along with the ability of TLR9 agonist to elevate CXCR4 levels warrants investigation regarding the use of TLR9 agonist as an effective anti-glioma target. Though pharmacologic inhibition of HIF-1α or the SDF-1/CXCR4 interaction abrogates regrowth of GBM [315], inhibition of HIF-1α reversed IGF-1 mediated decrease in CXCR4 expression. These findings suggested that HIF-1α regulates CXCR4 in different ways depending on the context of microenvironment.
Our findings have not only highlighted a previously unrecognized function of HIF-1α as an important regulator of SOCS3 and TLR9 expression, but has established HIF-1α as a link between two apparently unrelated but crucial component of glioma tumor microenvironment- growth factor and inflammation. Taken together, our data underline the complexity of HIF-1α in its crosstalk with TLR9 under normoxia. This study prompts further investigation into mechanisms governing the dialogue between HIF-1α and TLR9 to reveal new molecular components that participates in regulating inflammatory responses in glioma.

In vitro screening of compounds with anticancer properties by NCI identified Iridals for their anti-proliferative activity. Besides its ability to bind PKCα and RasGRP3 [248], nothing is known regarding the mechanism of action or bioavailability of Iripallidal. Our studies suggest that Iripallidal induce apoptosis in glioma cells and inhibits the Akt/mTOR pathway. The efficacy of mTOR inhibitors in glioblastoma cell lines [228, 230] has prompted their clinical trials for GBM [229, 316]. As rapamycin activates Akt pathway by a negative feedback loop involving phosphorylation of insulin receptor substrate (IRS) by mTOR effector molecule S6 kinase [317, 318], it was therefore not surprising that Rapamycin treatment induced Akt activation in some GBM patients in a Phase I clinical trial [229]. Moreover, dual inhibition of Akt and mTOR has proven effective in pre-clinical model of GBM [295], suggesting that dual Akt/ mTOR inhibitor can effectively overcome the effects of feedback loop efficiently than a single inhibitor selectively targeting mTOR. As mTOR blockade is a biomarker of therapeutic efficacy in glioma [319], the unique ability of Iripallidal to inhibit both Akt and mTOR can be exploited as novel anti-glioma therapy. In addition to inhibiting Akt/mTOR axis, Iripallidal also inhibited STAT3 signaling. PKC inhibitor attenuates Ras activation and this attenuation correlates with an inhibition of RasGRP3 phosphorylation [320]. Interestingly, PKCα regulates mTOR [319] as well as STAT3 activation [252]. It is possible that that Iripallidal effects Akt/mTOR and STAT3 signaling pathways through its ability to bind PKCα.
Iripallidal mediated decrease in STAT3 activation was concurrent with decreased cyclin D1 and increased p21 expression. While cyclin D1 overexpression and STAT3 activation are mutually exclusive events [321], p21 inhibits STAT3 signaling [322]. Besides, inhibition of mTOR signaling induces cell cycle arrest through regulation of Cyclin D and p27 [228]. As telomerase inhibition is known to cause apoptosis in human cancers [300], the ability of Iripallidal to down-regulate telomerase activity may also represent a mechanism for its anti-proliferative effect on glioma cells. Besides glioma cell lines, Iripallidal also decreased the viability of several other cancer cell types although to different extents. It is known that cytotoxic responses is a reflection of an integrated readout of all targets and/or biochemical pathways affected upon drug exposure [323]. As strong co-relation exists between chemoresponsiveness and gene expression [323], it is likely that differential expression of cellular pathways in cancer cell types of diverse origin could have resulted in differences in sensitivity to Iripallidal.

Taken together our studies suggest that (i) Iripallidal induces glioma cell apoptosis and (ii) inhibits Akt/ mTOR and STAT3 pathway. This ability of Iripallidal to act as a multi-inhibitor that blocks Akt/mTOR and STAT3 pathways suggest that its potential as a chemotherapeutic agent against GBM should be further evaluated. Also, Iripallidal effectively abrogated IGF-1 induced HIF-1α activation and pro-inflammatory cytokine response in glioma cell lines. As HIF-1α is considered to be one of the most important anti-cancer target [253], the potential ability of Iripallidal to regulate HIF-1α activation and inflammatory response in glioma warrants its investigation as a promising candidate for the treatment of GBM.