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SHORT COMMUNICATION

The p38-MK2-HuR pathway potentiates EGFRvIII–IL-1β-driven IL-6 secretion in glioblastoma cells

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The microenvironment of glioblastoma (GBM) contains high levels of inflammatory cytokine interleukin 6 (IL-6), which contributes to promote tumour progression and invasion. The common epidermal growth factor receptor variant III (EGFRvIII) mutation in GBM is associated with significantly higher levels of IL-6. Furthermore, elevated IL-1β levels in GBM tumours are also believed to activate GBM cells and enhance IL-6 production. However, the crosstalk between these intrinsic and extrinsic factors within the oncogene-microenvironment of GBM causing overproduction of IL-6 is poorly understood. Here, we show that EGFRvIII potentiates IL-1β-induced IL-6 secretion from GBM cells. Importantly, exacerbation of IL-6 production is most effectively attenuated in EGFRvIII-expressing GBM cells with inhibitors of p38 mitogen-activated protein kinase (p38 MAPK) and MAPK-activated protein kinase 2 (MK2). Enhanced IL-6 production and increased sensitivity toward pharmacological p38 MAPK and MK2 inhibitors in EGFRvIII-expressing GBM cells is associated with increased MK2-dependent nuclear–cytoplasmic shuttling and accumulation of human antigen R (HuR), an IL-6 mRNA-stabilising protein, in the cytosol. IL-1β-stimulated activation of the p38 MAPK–MK2-HuR pathway significantly enhances IL-6 mRNA stability in GBM cells carrying EGFRvIII. Further supporting a role for the p38 MAPK–MK2-HuR pathway in the development of inflammatory environment in GBM, activated MK2 is found in more than 50% of investigated GBM tissues and correlates with lower grade and secondary GBMs. Taken together, p38 MAPK–MK2-HuR signalling may enhance the potential of intrinsic (EGFRvIII) and extrinsic (IL-1β) factors to develop an inflammatory GBM environment. Hence, further improvement of brain-permeable and anti-inflammatory inhibitors targeting p38 MAPK, MK2 and HuR may combat progression of lower grade gliomas into aggressive GBMs.

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INTRODUCTION

Glioblastomas (GBM) are among the most lethal and least successfully treated solid tumours. These brain tumours contain high levels of inflammatory cytokines, in particular interleukins (IL) IL-1β, IL-6 and IL-8, which promote GBM proliferation, stemness, angiogenesis and invasion.[1]

Approximately 50% of all GBMs overexpress wild-type EGFR, often together with the truncated and constitutively active epidermal growth factor receptor variant III (EGFRvIII) mutant.[2] Oncogenic activity of EGFRvIII correlates with overproduction of IL-6[3] and is associated with upregulated EGFR effector Akt activity.[4] IL-6 is also produced by tumour-infiltrating microglia and astrocytes[5,6] and IL-1β, which is significantly elevated in GBM, most likely via enhanced mitogen-activated protein kinase (MAPK) signalling, promotes IL-6 production.[7,8] Thus, IL-6 production is triggered by multiple stimuli involving several tumour- and microenvironment-induced signalling events. However, the current understanding of IL-6 synthesis in GBM is almost exclusively based on models utilising cancer or glial cells in isolation, not incorporating the contribution of tumour-microenvironment interactions.

IL-6 expression is tightly regulated by extrinsic and intrinsic signals targeting transcription factors like cAMP response element-binding protein (CREB) and activating transcription factor 1 (ATF-1) to control IL-6 promoter activity.[7] In addition, IL-6 mRNA is characterised by rapid turnover, which is controlled by cellular factors, including human antigen R (HuR).[7,9] Upon cell stimulation, HuR monomer in the nucleus undergoes dimerisation and translocates to the cytoplasm, where HuR dimer binds and stabilizes its target mRNA.[10] The nuclear–cytoplasmic shuttling of HuR is critical for HuR-dependent mRNA stabilisation, and is regulated via several signalling pathways.[11] Most interestingly, activation of p38 mitogen-activated protein kinase (p38 MAPK), possibly via IL-1β and tumour necrosis factor-α (TNF-α), is implicated in HuR-triggered stabilisation of mRNA coding for key inflammatory mediators, including IL-6, IL-8, cyclooxygenase-2 (COX-2) and TNF-α.[12–15] p38 MAPK-induced activation of MAPK-activated protein kinase 2 (MAPK-APK2 or MK2) appears crucial for HuR translocation and stabilisation of target mRNAs.[16,17] In non-stimulated cells, MK2 and p38 MAPK exist as a preformed inactive complex in the nucleus. After activation by upstream kinases, for example, MAPK kinase 6, p38 MAPK phosphorylates MK2 at threonine 334 (T334), which enables the p38 MAPK–MK2 complex

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translocation. MK2 T334 phosphorylation exposes the MK2 substrate-binding site, which might potentiate HuR translocation kinetics.[18] Therefore, inhibition of the p38 MAPK–MK2 pathway could interfere with cytoplasmic HuR accumulation, which is rapidly developing into a new marker and potential hallmark of cancer cells.[19–21]

Here we investigated IL-6 synthesis and secretion in the oncogene-microenvironment context. Inflammatory cytokines, such as IL-1β and TNF-α, show enhanced potential to upregulate IL-6 secretion in EGFRvIII-expressing GBM cells. In these cells, augmented potency of p38 MAPK and MK2 inhibitors to block IL-6 secretion is associated with an increased involvement of p38 MAPK and MK2 in nuclear–cytoplasmic HuR translocation and IL-6 mRNA stabilisation. In addition, the oncogenic EGFRvIII mutant alters expression and activity of key regulators and binding partners of HuR. Identification of activated MK2 in GBM tumours, in particular lower grade and secondary GBMs, implicate the p38-MK2-HuR pathway as a target to inhibit the development of an inflammatory environment in GBM.

RESULTS AND DISCUSSION

Oncogenic EGFRvIII upregulates basal secretion of IL-6 and IL-8 in unstimulated GBM cells.[3,22] To address if EGFRvIII could enhance IL-6 secretion in GBM cells upon extrinsic stimulation, we treated U87 and U87-EGFRvIII cells with increasing amounts of IL-1β (0–100 ng/ml) and compared IL-6 secretion (Figure 1a). IL-1β stimulated U87-EGFRvIII consistently secreted approximately four-fold higher quantities of IL-6 when compared with U87 cells. Increasing concentrations of IL-1β also elevated IL-8 secretion in U87-EGFRvIII cells (Supplementary Figure 1A).

p38 MAPK inhibitors efficiently reduce IL-6 secretion in IL-1β-treated U251 GBM cells.[8] To investigate the role of p38 MAPK–MK2 signalling for IL-6 secretion in U87 and U87-EGFRvIII cells, cells were pre-incubated with p38 MAPK inhibitor SB203580 (SB) or MK2 inhibitor, compound sc-221948 (thereafter sc-48, Figure 1b). IL-1β-induced IL-6 secretion in U87 cells was reduced by 27.6 ± 4.3% with SB203580; however, this reduction was not statistically significant. SB203580 was much more potent to inhibit IL-1β-induced secretion of IL-6 from U87-EGFRvIII cells (68.2 ± 15.7%). Similarly, IL-6 secretion in IL-1β-incubated U87-EGFRvIII cells was more effectively inhibited with the MK2 inhibitor sc-48 as compared with controls (68.2 ± 18.8% vs 6.8 ± 1.1%). Augmented susceptibility of U87-EGFRvIII cells toward SB203580 was not due to increased SB203580 internalisation (Supplementary Figures 1B and C).

To address the contribution of other EGFR effector pathways[23] in IL-1β-induced IL-6 secretion, we next compared IL-6 secretion upon incubation with p38 MAPK (069A), MK2 (sc-38), MAPK kinase Mek1/2 (PD098059) and c-Jun N-terminal kinase (JNK) (SP600125) inhibitors (Figure 1c). As above, p38 MAPK and MK2 inhibitors were significantly (>threefold) more effective in attenuating IL-1β-induced IL-6 secretion in U87-EGFRvIII cells. Most strikingly, Mek1/2 or JNK inhibition did not downregulate IL-6 secretion in U87 and U87-EGFRvIII cells.

We next compared p38 MAPK, Mek1/2 and JNK inhibitors to block TNF-α and IL-1β-induced IL-6 and IL-8 expression (Supplementary Table 1). In all settings, Mek1/2 and JNK inhibitors reduced IL-6 and IL-8 secretion with similar efficacy in U87 and U87-EGFRvIII cells or were even less effective in EGFRvIII-expressing cells. Hence, oncogenic EGFR does not seem to alter the contribution of Mek1/2 and JNK in IL-6 and IL-8 secretion from IL-1β- or TNF-α-stimulated GBM cells. Interestingly, only IL-1β- and TNF-α-induced IL-6, but not IL-8, secretion was highly susceptible to p38 MAPK and MK2 inhibition in U87-EGFRvIII cells (Supplementary Table 1 and Figures 1b and c), strongly indicating that the p38 MAPK–MK2 pathway specifically targets molecular events controlling some, but not all members of the IL family.

p38 MAPK signalling has been linked to the trafficking of secretory vesicles.[24,25] p38 MAPK inhibition reduced both intra- and extracellular IL-6 levels much more efficiently in U87-EGFRvIII (83.4 ± 8.0%) compared with U87 cells (51.3 ± 11.4%, Supplementary Figure 1D). Taken together, p38 MAPK and MK2 inhibitors more efficiently inhibit inflammatory IL-6 cytokine production in IL-1β-treated GBM cells expressing EGFRvIII.

We next compared IL-6 mRNA expression of IL-1β-incubated U87 and U87-EGFRvIII cells ± SB203580 using RT–PCR (Figure 1d). In line with increased IL-6 secretion (Figure 1a), basal (1.9 ± 0.6-fold) and IL-1β-induced IL-6 mRNA levels in U87-EGFRvIII cells were increased compared with controls (139.0 ± 8.5-fold and 40.8 ± 19.6-fold, respectively; Figure 1d). SB203580 significantly reduced IL-6 mRNA levels upon IL-1β stimulation by 34.0 ± 11.2% and 82.9 ± 4.2% in U87 and U87-EGFRvIII cells, respectively. By contrast, p38 MAPK inhibition did not reveal increased potency to reduce IL-1β-induced IL-8 mRNA levels in U87-EGFRvIII cells compared with controls (Supplementary Figure 1E). Hence, increased potency of SB203580 to block cytokine production in EGFRvIII cells specifically affect IL-6 mRNA levels.

Then we compared the phosphorylation kinetics ($t = 0$–120 min) of p38 MAPK, MK2 and heat-shock protein 27 (Hsp27), a downstream target of MK2, in IL-1β-stimulated U87 and U87-EGFRvIII cells (Supplementary Figure 2). However, p38 MAPK, MK2 and Hsp27 phosphorylation kinetics were similar in U87 and U87-EGFRvIII cells, indicating that EGFRvIII or IL-1β-induced EGFR wild-type transactivation[26] did not impact on p38 MAPK-dependent MK2 and Hsp27 phosphorylation.

To address efficacy of p38 MAPK and MK2 inhibitors, we analysed p38 MAPK and MK2 phosphorylation ± SB203580 and sc-48. As above, p38 MAPK activation by IL-1β was comparable in U87 and U87-EGFRvIII cells (Figure 2a, lanes 2 and 5). In line with published data,[27] SB203580 attenuated IL-1β-induced p38 MAPK phosphorylation by 49.9 ± 11.8% in U87 cells (lanes 2–3) and by 75.6 ± 17.9% in U87-EGFRvIII cells (lanes 5–6). Despite increased potency of SB203580 to inhibit p38 MAPK phosphorylation in U87-EGFRvIII cells, this did not translate into increased inhibition of p38 MAPK activity, as SB203580 completely blocked MK2 phosphorylation in both U87 and U87-EGFRvIII cells (lanes 3 and 6).

We then addressed MK2 inhibitor sc-48 efficacy. U87 and U87-EGFRvIII cells were pre-treated with sc-48 and stimulated with IL-1β for 15 min. As sc-48 only moderately inhibits MK2 activation, but attenuates MK2 kinase activity,[28] we analysed phosphorylation of the MK2 substrate Hsp27. In line with data shown above, IL-1β-induced MK2 phosphorylation was evident in both cell lines, and was not downregulated with sc-48 (Figure 2b, lanes 3 and 6). Hsp27 phosphorylation ± IL-1β and MK2 inhibitor sc-48 were comparable in U87 and U87-EGFRvIII cells (lanes 2, 3 and 5, 6). Taken together, inhibition of p38 MAPK and MK2 more potently reduced IL-6 secretion in IL-1β-activated U87-EGFRvIII cells (Figure 1), yet magnitude of p38 MAPK, MK2 and Hsp27 inhibition, as judged by phosphorylation, appeared comparable in U87 and U87-EGFRvIII cells (Figure 2).

To investigate this further, we examined CREB and ATF-1, downstream targets of p38 MAPK that regulate IL-6 promoter activity. However, high CREB and ATF-1 phosphorylation, indicative of upregulated CREB/ATF-1 activity, was evident in control and IL-1β-incubated cells (Supplementary Figure 3A). In addition, SB203580 did not significantly attenuate IL-6 promoter-driven luciferase activity in IL-1β-stimulated U87 and U87-EGFRvIII cells (Supplementary Figure 3B). Taken together, the p38 MAPK–MK2 pathway does not seem to play a major role in the transcriptional regulation of IL-6 expression in U87 cells.

We next studied the involvement of the p38 MAPK–MK2 pathway in HuR-dependent post-transcriptional regulation of IL-6 mRNA expression (Figure 3a). Indeed, siRNA-targeting HuR effectively downregulated HuR levels in U87 (87.4 ± 0.1%) and U87-EGFRvIII cells (82.6 ± 2.6%). HuR knockdown was associated

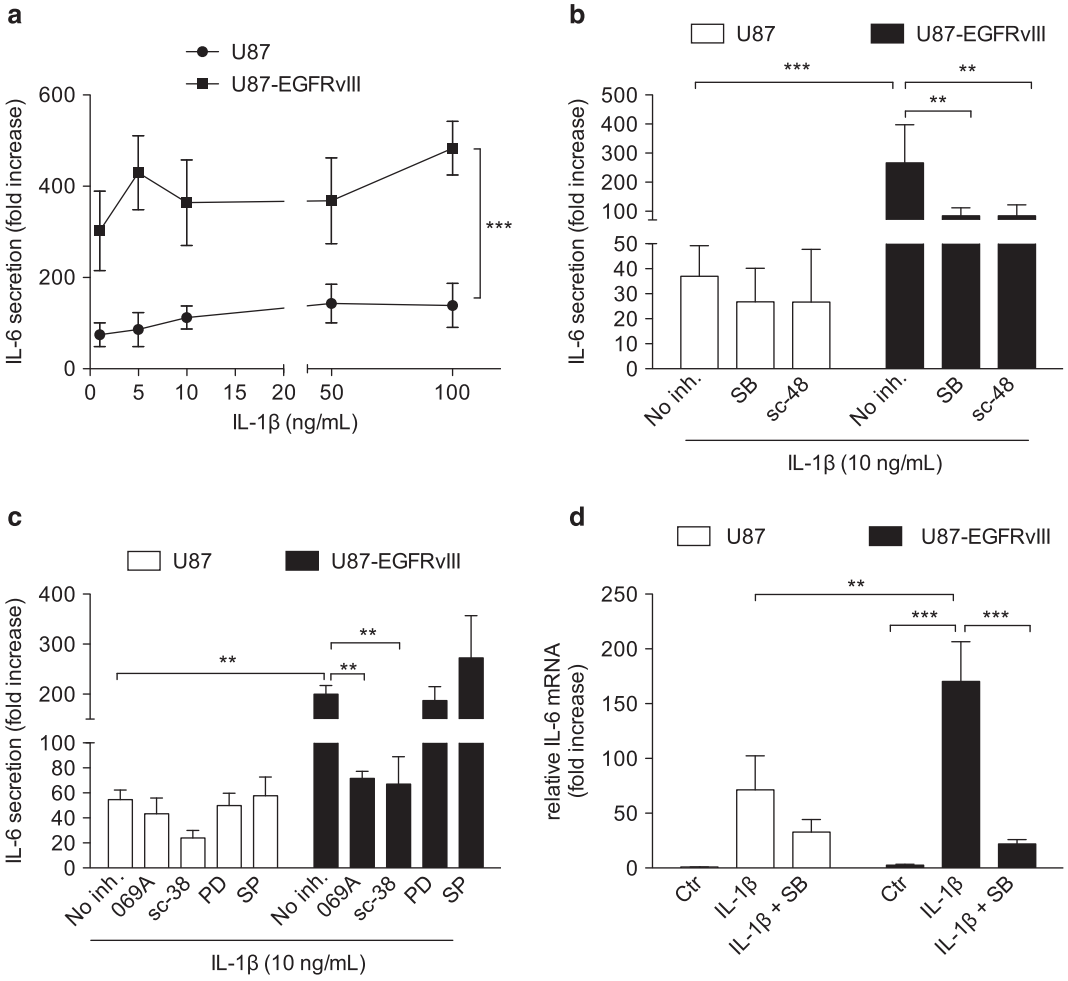


Figure 1. Inhibition of p38 MAPK–MK2 signalling attenuates IL-6 secretion in glioblastoma cells. (**a**) U87 and U87-EGFRvIII (5×10^4) cells were treated with IL-1β (0–100 ng/ml) for 24 h and secretion of IL-6 was determined by enzyme-linked immunosorbent assay (ELISA). Values were normalised to cell viability (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) and expressed as fold increase (mean ± s.e.m.) compared with untreated U87 cells. (**b–c**) U87 and U87-EGFRvIII (5×10^4) cells were starved for 2 h, pre-treated with compounds (10 μM) inhibiting (**b**) p38 MAPK (SB203580, SB), MK2 (sc-221948, sc-48) or (**c**) p38 MAPK (069A,[36]), MK2 (sc-203138, sc-38 1 μM), MEK1/2 (PD98059, PD) or JNK (SP600125, SP) for 60 min and treated with IL-1β (10 ng/ml) for 24 h. Secretion of IL-6 was determined using IL-6 ELISA kit and normalised to cell viability as above. See Supplementary Material for further details. (**d**) U87 and U87-EGFRvIII (3×10^5) cells were starved for 2 h, pre-treated with the 10 μM SB203580 (SB) and treated with IL-1β (10 ng/ml) for 24 h. IL-6 mRNA was determined by RT–PCR and normalised to 18 s mRNA (for details see Supplementary Material and Munoz L *et al.*[37]) data are expressed as fold increase compared with untreated cells. All data are mean ± s.e.m. from at least three independent experiments performed in duplicates. (**$P < 0.01$, ***$P < 0.001$, one-way analysis of variance followed by Newman–Keuls post-test using Prism 5 GraphPad Software).

with 56.5 ± 3.4% reduced IL-6 production in IL-1β-stimulated U87 cells (Figure 3a). Moreover, HuR knockdown in IL-1β-treated U87-EGFRvIII cells decreased IL-6 secretion by 76.3 ± 3.0% (Figure 3a), indicating increased involvement of HuR in IL-6 mRNA stabilisation in EGFRvIII.

Accumulation of cytoplasmic HuR may be common in tumours, including GBM.[20,21] To possibly identify a link between oncogenic EGFR and nuclear–cytoplasmic HuR shuttling, U87 and U87-EGFRvIII cells were harvested and whole-cell lysates were subjected to subcellular fractionation. Both cell lines express comparable amounts of HuR (Figure 3c). Non-malignant primary human astrocytes served as control. Nuclear fractions, enriched with nuclear marker lamin A/C, and cytosolic fractions were prepared (Figure 3b). Activated HuR forms a stable dimer,[10] hence reducing conditions were used to ensure HuR monomer (37 kDa) analysis. Consistent with published data, HuR was predominantly found in nuclear fractions of primary human astrocytes (lane 2). In U87 GBM cells, increased quantities of HuR were evident in the cytoplasmic fraction (lane 3). Most strikingly, in U87-EGFRvIII cells, HuR accumulated in the cytoplasm (72.1 ± 5.4%, lane 5). These findings suggested that EGFRvIII may enhance HuR translocation in GBM cells.

In order to explain increased cytosolic HuR accumulation in U87-EGFRvIII cells, we compared expression and activation of several HuR-interacting proteins in U87 and U87-EGFRvIII cells (Figure 3c). Total amounts of p38 MAPK and MK2 in U87 and U87-EGFRvIII were comparable (Figure 3c and Supplementary Figure 4A). Slightly elevated expression of MK2 was found in EGFRvIII-expressing cells, but the levels did not reach statistical significance when compared with controls. Intriguingly, U87-EGFRvIII cells showed a twofold difference in the expression and inactivation of cyclin-dependent kinase 1 (Cdk1). Cdk1 is a cell cycle kinase that is inactivated through phosphorylation in the event of DNA damage, causing G_2 arrest.[29] Importantly, HuR shuttling is controlled by the Cdk1-mediated phosphorylation of Ser202 that acts to retain HuR in the nucleus.[30] Increased Cdk1 inactivation, most likely due to the EGFRvIII-mediated replicative stress and DNA damage causing G_2 arrest,[31] correlated with

decreased levels of pHuR(S202) in U87-EGFRvIII (Figure 3c) and increased cytosolic HuR accumulation (Figure 3b). It is important to note that only unphosphorylated HuR shuttles into the cytosol and pHuR(S202) could not be detected in the cytosolic fractions. This mechanism could contribute to increase HuR accumulation in the cytoplasm and thereby increase efficacy of p38 MAPK and MK2 inhibitors, as p38 MAPK–MK2 signalling is activated by DNA damage and causes inactivation of Cdk1 and G_2 arrest.[29] In addition, we found that U87-EGFRvIII cells express significantly more chromosome region maintenance 1 (CRM1/exportin 1) protein (Figure 3c). CRM1 is a ubiquitous transport receptor that recognises the nuclear export sequence of activated MK2 and

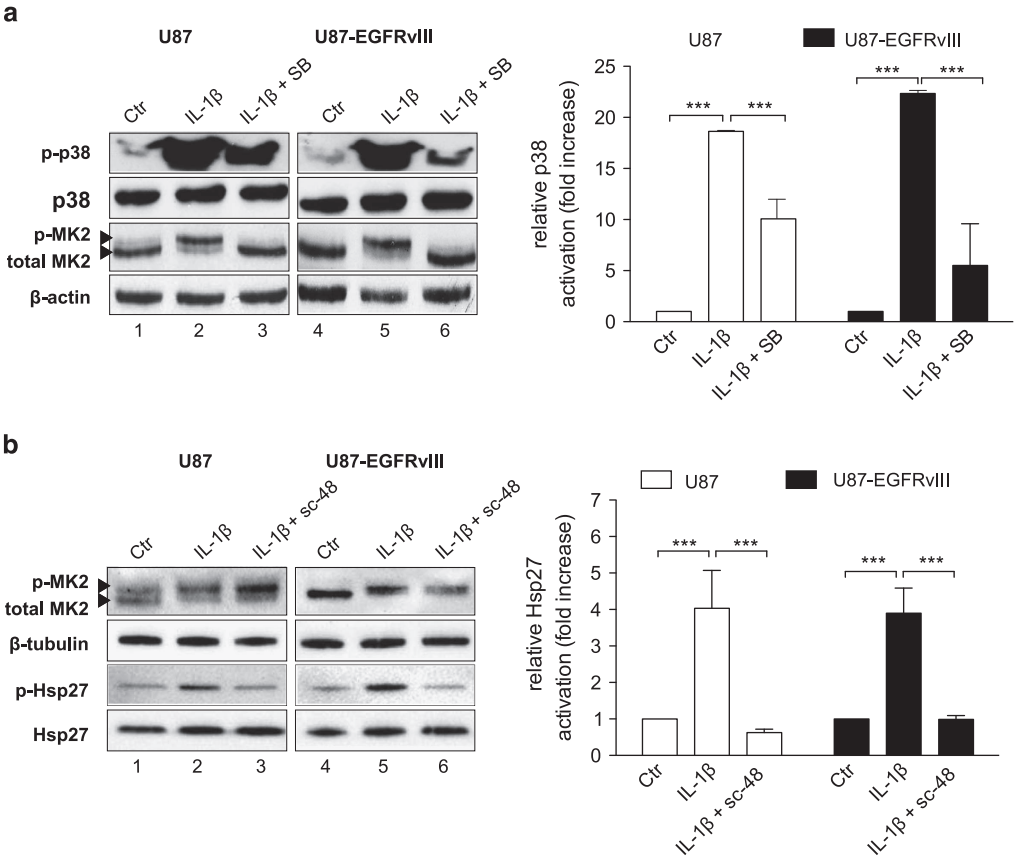


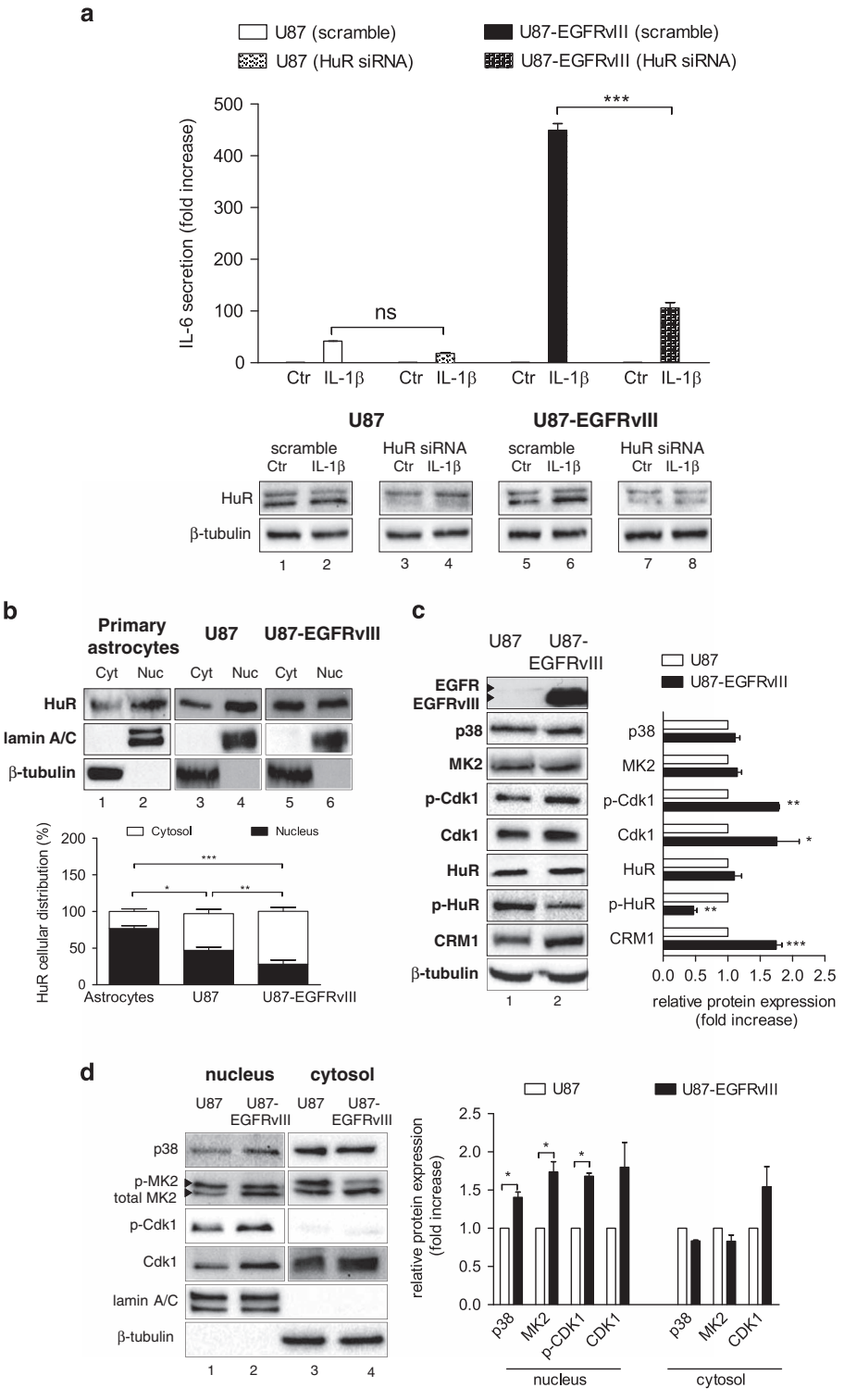
Figure 2. IL-1β activates the p38 MAPK–MK2 pathway in U87 and U87-EGFRvIII cells. (**a–b**) U87 and U87-EGFRvIII (4×10^5) cells were starved for 2 h, pre-treated with (**a**) p38 MAPK inhibitor SB203580 (SB, 10 μM) or (**b**) MK2 inhibitor sc-48 (10 μM) for 60 min and treated with IL-1β (10 ng/ml) for 15 min. Protein concentration of whole-cell lysates was determined and 25 μg cellular protein was subjected to western blot analysis as described (Yeung Y *et al.*[8], see Supplementary Material) and analysed for p38 MAPK phosphorylation (p-p38), total p38 MAPK (p38), phosphorylated MK2 (p-MK2), total MK2 (MK2), phosphorylated Hsp27 (p-Hsp27), total Hsp27 (Hsp27), β-actin and β-tubulin (all Cell Signalling) as indicated. Relative levels of p-p38 and p-Hsp27 were normalised to total p38 and total Hsp27, respectively, expressed as fold increase compared with untreated (Ctr) cells. Representative blots and quantification of three (**a**) and five (**b**) independent experiments are shown. Data represent the mean ± s.e.m. (***$P < 0.001$, one-way analysis of variance followed by Newman–Keuls post-test using Prism 5 GraphPad Software).

Figure 3. HuR is critical for IL-6 secretion in U87 glioblastoma cells. (**a**) U87 and U87-EGFRvIII (2×10^5) cells were transfected with siRNA targeting HuR (5′-ACUUAUUCGGGAUAAAGUATT-3′, 5′-UACUUUAUCCCGAAUAAGUTT-3′) (for details see Supplementary Material). Scrambled siRNA served as control. After 72 h, cells were stimulated with IL-1β (10 ng/ml) for 24 h. IL-6 secretion was determined by enzyme-linked immunosorbent assay (ELISA) as described above. Whole-cell lysates were subjected to western blot analysis to confirm HuR knockdown (87.4 ± 0.1% and 82.6 ± 2.6% in U87 and U87-EGFRvIII cells, respectively). Representative blot and data analysis (mean ± s.e.m.; ***$P < 0.001$, one-way analysis of variance followed by Newman–Keuls post-test) of three independent experiments is shown. (**b**) U87 and U87-EGFRvIII (1×10^7) cells were subjected to subcellular fractionation (see Supplementary Material for details). Primary human astrocytes served as control in **b** and were isolated as described.[38] Protein concentrations of nuclear and cytosolic fractions were determined and 15–25 μg of total protein was subjected to western blot analysis. Cellular distribution of HuR, nuclear marker lamin A/C and β-tubulin was analysed. Nuclear and cytosolic HuR distribution was quantified and normalised to lamin A/C or β-tubulin, respectively. Representative blots and data (mean ± s.e.m., *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, one-way analysis of variance followed by Newman–Keuls post-test) from three independent experiments is shown. (**c**) Whole-cell lysates from unstimulated U87 ± EGFRvIII (40 μg) were subjected to western blot analysis and analysed for EGFR and EGFRvIII, p38 MAPK (p38), total MK2 (MK2), phosphorylated Cdk1 (p-Cdk1), total Cdk1 (Cdk1), total HuR (HuR), phosphorylated HuR (pHuR (S202),[30] CRM1 and β-tubulin (all Cell Signalling) as indicated. Representative blots and data (mean ± s.e.m., *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, paired Student's *t*-test) from three independent experiments are shown. (**d**) Nuclear (15 μg) and cytosolic (30 μg) fractions from U87 and U87-EGFRvIII were prepared and analysed by western blotting for total p38 MAPK (p38), phosphorylated and total MK2 (p-MK2 and MK2, respectively), phosphorylated and total Cdk1 (p-Cdk1 and Cdk1, respectively), β-tubulin and lamin A/C. Protein expression was quantified and normalised against lamin A/C (nuclear fractions) and β-tubulin (cytosolic fraction), expressed as fold increase compared with U87. Representative blots and data (mean ± s.e.m., *$P < 0.05$; paired Student's *t*-test) from three independent experiments are shown.

exports MK2-complexes into the cytoplasm.[32] Thus, increased CRM1 expression may enhance MK2-HuR export from the nucleus of U87-EGFRvIII cells, where MK2 is predominantly located.

Next, the subcellular distribution of p38 MAPK and MK2 was analysed (Figure 3d). U87 cells showed less nuclear p38 MAPK (lane 1) compared with U87-EGFRvIII cells (lane 2) and MK2 also appeared enriched in nuclear fractions from U87-EGFRvIII cells (lane 2). Similarly, expression and phosphorylation of Cdk1 were enhanced in the nuclear fractions from EGFRvIII-expressing cells (lane 2). Together, this data suggested that increased nuclear localisation of several HuR-interacting proteins, in particular MK2, in U87-EGFRvIII cells improves signal efficacy to activate and promote nuclear HuR translocation. These changes in subcellular p38 MAPK, MK2 and HuR distribution correlate with increased efficacy of SB203580 and sc-48 to attenuate IL-6 secretion in these cells.

As IL-1β induces nuclear HuR export[33] and MK2 facilitates HuR shuttling,[34] we investigated if MK2 inhibition could attenuate



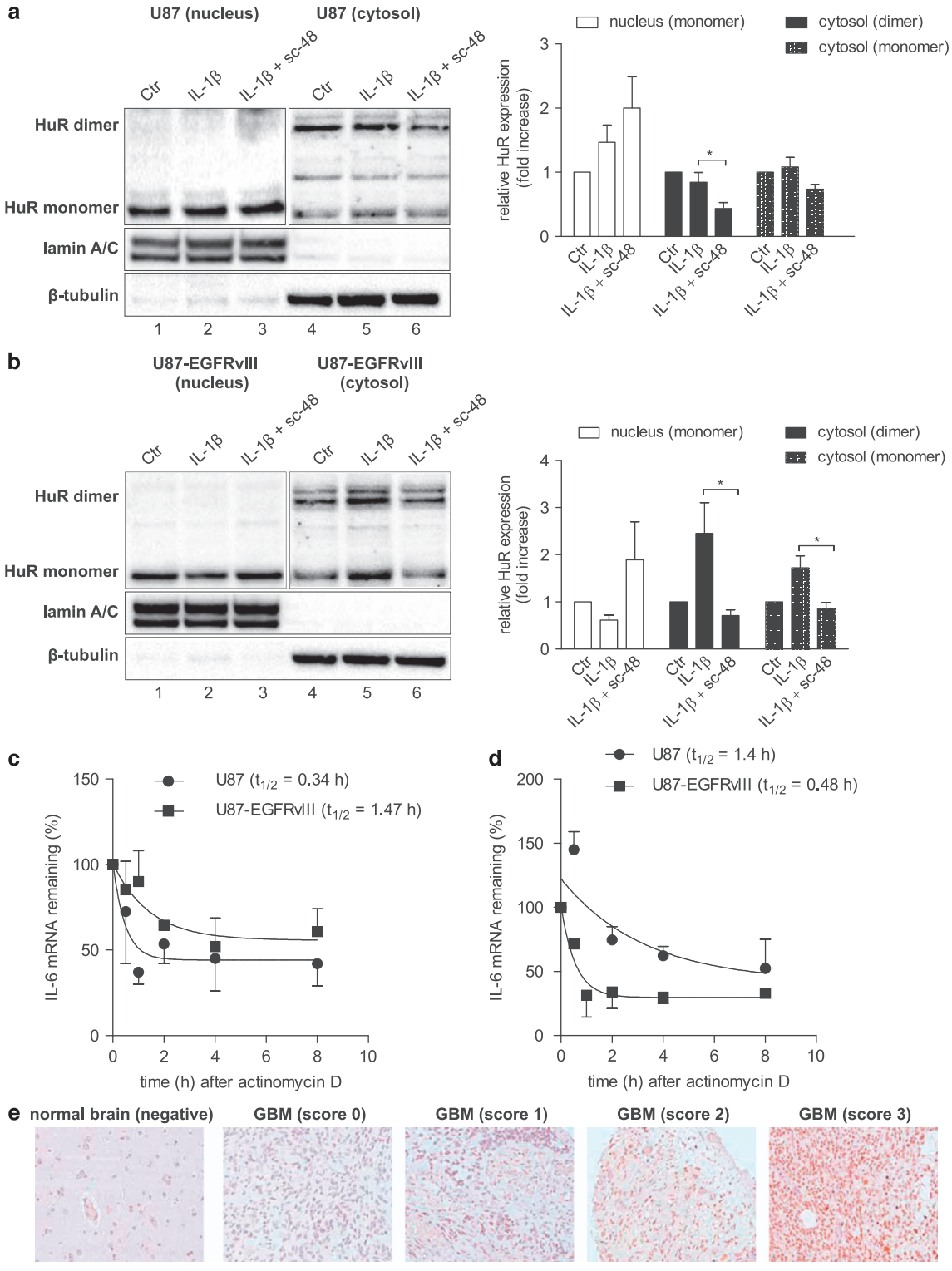


Figure 4. MK2 inhibition attenuates nuclear–cytoplasmic translocation of HuR and IL-6 mRNA stability in U87 cells. (**a–b**) U87 and U87-EGFRvIII (1×10^7) cells were starved for 2 h, pre-treated with sc-48 (10 μM) for 60 min and activated with IL-1β (10 ng/ml) for 8 h. Nuclear and cytosolic fractions were prepared and analysed by western blotting for the distribution of HuR, lamin A/C and β-tubulin (for details see Supplementary Material). Relative HuR levels were quantified and normalised to lamin A/C (nuclear fractions) or β-tubulin (cytosolic fractions). Representative blots and data (mean ± s.e.m., $^*P < 0.05$, paired Student's *t*-test) from three independent experiments are shown. (**c–d**) U87 and U87-EGFRvIII (4×10^5) cells were starved for 2 h, pre-treated with sc-48 (10 μM) for 60 min (**d**) and stimulated with IL-1β (10 ng/ml) for 4 h (**c–d**). Cells were then washed and incubated with actinomycin D (5 μg/ml). Total RNA was extracted at indicated timepoints and IL-6 mRNA quantified. Results are expressed as % mRNA remaining over time (for details see Supplementary Material). Data are mean ± s.e.m. from three independent experiments. (**e**) Representative images of GBM tissues stained with antibody recognising activated MK2 (pT334-MK2) (for details see Supplementary Material).

Table 1. Formalin-fixed, paraffin-embedded tissues from 205 brain tumour patients with grade II–IV astrocytomas who underwent surgery in the Unit of Neurosurgery, Tampere University Hospital, Tampere, Finland, during 1983–2001 were used for the construction of TMA

		Relative p-MK2 expression			
Score		*0*	*1*	*2*	*3*
Histological type	N	n *(%)*	n *(%)*	n *(%)*	n *(%)*
Astrocytoma (WHO grade 2)	25	8 (32)	3 (12)	12 (48)	2 (8)
Anaplastic astrocytoma (WHO grade 3)	31	10 (32)	2 (6)	15 (48)	4 (13)
Glioblastoma (WHO grade 4)	149	78 (52)	11 (7)	52 (35)	8 (5)
Subtotal		96	16	79	14
Total	205	96 (47%)		109 (53%)	

	N	*p-MK2 negative (< 5% positive cells)* n *(%)*	*p-MK2 positive (> 5% positive cells)* n *(%)*	*Significance (χ^2-test)*
Primary GBM	119	63 (53)	56 (47)	$P = 0.02$
Secondary GBM	5	0 (0)	5 (100)	
IDH-1 (negative)	115	66 (57)	49 (43)	$P = 0.002$
IDH -1 (positive)	44	13 (30)	31 (70)	
p53 (negative)	48	30 (62)	18 (38)	$P = 0.025$
p53 (positive)	48	19 (39)	29 (61)	
EGFR (no amplification)	92	42 (46)	50 (54)	
EGFR (amplification)	44	26 (59)	18 (41)	n.s.

Abbreviations: EGFR, epidermal growth factor receptor; GBM, glioblastoma; IDH, isocitrate dehydrogenase; n.s., non significant; p-MK-2, phosphorylated MK2; TMA, tissue microarrays; WHO, World Health Organization. The study was approved by the Ethical Review Board of Tampere University Hospital, Tampere, Finland (Dnro R07042), the National Authority for Medicolegal Affairs of Finland (Dnro 1502/04/046/07) and the Human Ethics Committee of the University of Sydney (HREC 2013/131). Activated MK2 (p-MK2) was detected with rabbit polyclonal antibody recognising phosphorylated MK2 (T334, Cell Signaling Technology, Beverly, MA, USA, for details see Supplementary Material). Tissue sections were counterstained with haematoxylin, and p-MK2 immunoreactivity was evaluated by two pathologists using a consultation microscope (for representative images, see Figure 4e).

IL-1β-induced nuclear export of HuR in GBM cells. Therefore U87 and U87-EGFRvIII were pre-treated with sc-48, followed by incubation with IL-1β for 8 h (Figure 4). As HuR activation involves cysteine-dependent dimerisation, non-reducing conditions to detect HuR monomers (~37 kDa) and dimers (~70 kDa) were chosen (for details see Supplementary Material). The identity of the band corresponding to HuR dimer was confirmed by its sensitivity toward HuR depletion in immunoblots from HuR knockdown lysates (Supplementary Figure 4B). Monomeric HuR was found in the nucleus of unstimulated and IL-1β ± sc-48-incubated U87 cells (Figure 4a, lane 1–3). Since HuR is abundant in the nucleus, but under normal or non-stimulated conditions almost undetectable in the cytosol, HuR shuttling is generally based on analysis of cytoplasmic fractions.[30] Nevertheless, we observed a 1.5-fold increase in monomeric HuR nuclear levels in IL-1β-treated U87 cells (Figure 4a, lane 1–2). Comparable amounts of HuR monomer and dimer were detected in the cytosol of U87 cells in the presence or absence of IL-1β (Figure 4a, lanes 4–5). This suggests that IL-1β increases HuR expression (see also Figure 3a), without increasing HuR cytoplasmic accumulation. Importantly, MK2 inhibitor sc-48 significantly reduced cytosolic HuR monomers and dimers in U87 cells (lane 6), which was accompanied by a twofold increase in nuclear HuR (lane 3). Hence, blocking MK2-dependent HuR cytoplasmic accumulation could reduce IL-6 expression in U87 cells.

Most strikingly, U87-EGFRvIII cells treated with IL-1β displayed a 0.5-fold decrease in nuclear HuR and 2.5-fold HuR increase in the cytosolic fraction (Figure 4b, lanes 2 and 5). As observed in U87 cells, MK2 inhibition by sc-48 effectively reduced cytosolic HuR (see decreased monomer and dimer in lane 6), which was accompanied by a twofold increased nuclear amounts of HuR monomers (lane 3).

To further explore the molecular mechanism underlying enhanced HuR shuttling in EGFRvIII-expressing cells after IL-1β treatment, we compared the localisation of MK2 and phosphorylation state of HuR in U87 cells ± EGFRvIII (Supplementary Figure 5A–B). In U87 cells, MK2 is primary localised in the cytosol (Supplementary Figure 5B, lane 1) and upon IL-1β stimulation, activated MK2 was distributed in both nuclear (Supplementary Figure 5A, lanes 2–3) and cytosolic fractions (Supplementary Figure 5B, lanes 2–3; for quantification see bottom panels). In addition, IL-1β stimulation of U87 cells did not significantly increase phosphorylation-dependent inactivation of nuclear Cdk1 and consistent with these observations, pHuR(S202) levels remained unchanged in these cells (Supplementary Figure 5A, lanes 1–3). Consistent with data shown above (Figure 3d), in unstimulated U87-EGFRvIII cells increased amounts of MK2 were found in the nucleus (Supplementary Figure 5A, lane 4) and activated p-MK2 remained localised in the nucleus upon IL-1β stimulation (lanes 5–6), whereas low p-MK2 levels were detected in the cytoplasmic fractions (Supplementary Figure 5B, lanes 5–6). Enhanced nuclear accumulation of activated MK2 was accompanied by significant phosphorylation-dependent inactivation of Cdk1 (Supplementary Figure 5A, lanes 5–6) and 0.63-fold decrease in pHuR(S202) levels (Supplementary Figure 5A, lanes 6). Taken together, this could explain the lack of increased cytoplasmic HuR levels in IL-1β-stimulated U87 cells compared with U87-EGFRvIII cells (Figure 4a). Similarly, p38 MAPK inhibitor SB203580 was more effective in preventing nuclear export of HuR in U87-EGFRvIII cells (Supplementary Figure 5C). In summary, MK2 accumulation in the nucleus and increased nuclear–cytosolic shuttling of HuR could be responsible for enhanced IL-6 production in IL-1β-stimulated U87-EGFRvIII cells.

To address if inhibition of HuR shuttling by MK2 inhibitor sc-48 altered IL-6 mRNA stability, U87 ± EGFRvIII cells were pre-treated ± sc-48 (10 μM) and stimulated with IL-1β for 4 h. Transcription was then halted using actinomycin D, and real-time RT–PCR was used to measure IL-6 mRNA degradation over time to determine the kinetics of IL-6 mRNA decay. In the absence of sc-48, IL-6 mRNA decay was slower in IL-1β-treated U87-EGFRvIII cells (decay constant $k = 0.66 \pm 0.2$; $t_{1/2} = 1.47 \pm 0.4$ h) compared with U87 cells (decay constant $k = 1.88 \pm 0.4$; $t_{1/2} = 0.34 \pm 0.1$ h, Figure 4c).

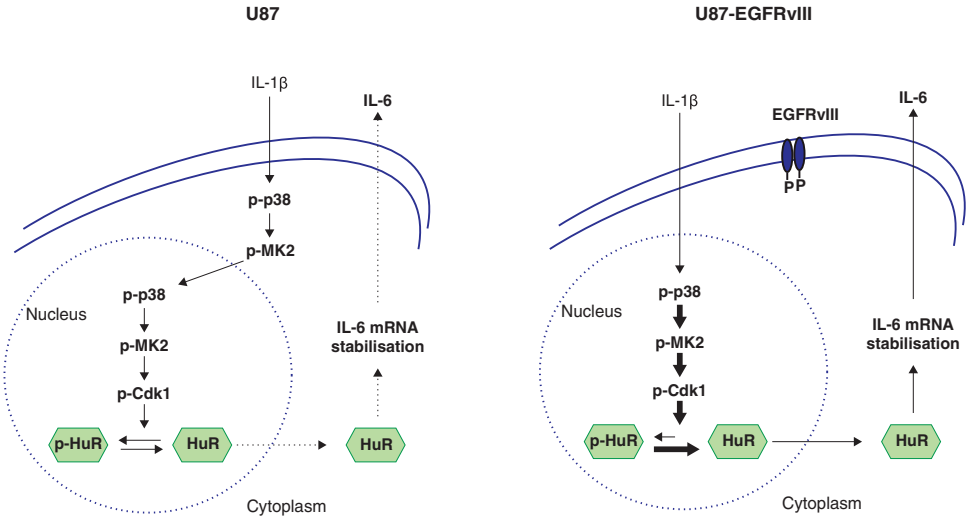


Figure 5. Proposed model of the molecular mechanism underlying IL-6 production in an inflammatory microenvironment with elevated IL-1β levels stimulating glioblastoma cells with and without EGFRvIII. In U87 cells, MK2 is primarily located in the cytosol. Upon IL-1β treatment, the activated p38 MAPK–MK2 complex translocates to the nucleus, which leads to phosphorylation-dependent inactivation of Cdk1. This process is amplified in U87-EGFRvIII cells where MK2 is predominantly located in the nucleus. This potentiates Cdk1 phosphorylation and consequently, increased amounts of inactive nuclear Cdk1 shift the phosphorylation equilibrium of HuR toward reduction of phosphorylated HuR (pHuR S202) levels. Importantly, only unphosphorylated HuR is able to translocate into the cytosol of U87-EGFRvIII after IL-1β treatment. In the cytoplasm, HuR stabilises IL-6 mRNA levels, leading to increased IL-6 protein production and secretion.

As shown in Figure 4d, sc-48 reduced IL-1β-induced IL-6 mRNA stability in U87-EGFRvIII cells (decay constant $k = 2.1 \pm 0.9$; $t_{1/2} = 0.48 \pm 0.2$ h), but increased IL-6 mRNA stability in U87 cells (decay constant $k = 0.55 \pm 0.1$; $t_{1/2} = 1.43 \pm 0.7$ h). Increased involvement of HuR in IL-6 mRNA stabilisation in U87-EGFRvIII cells may increase sensitivity toward the MK2 inhibitor sc-48, which as shown here interferes with HuR shuttling, IL-6 mRNA stability and IL-6 secretion (Figures 4b–d, Figure 1b).

Our data indicated that MK2 activation may be associated with brain tumour progression. We therefore compared p-MK2 staining in glioma tumours ($n = 205$) of different grade (Table 1). Non-malignant brain samples ($n = 6$) served as control and all showed no p-MK2 immunoreactivity (Figure 4e). In diffuse astrocytomas (WHO (World Health Organization), grade 2, $n = 25$), 68% tumours showed weak, moderate or strong p-MK2 staining in more than 5% cells. In anaplastic astrocytomas (WHO grade 3, $n = 31$), 68% tumours were classified as positive for p-MK2. In the most aggressive GBM cohort (WHO grade 4, $n = 149$), 48% tumours showed weak, moderate or strong staining for p-MK2 (Table 1 and Figure 4e). Statistical association of MK2 activation with lower WHO grade gliomas was observed ($P = 0.036$, χ^2 test). Lower grade gliomas often develop into secondary GBMs. Analysis of all secondary GBMs ($n = 5$) showed strong staining for p-MK2. Furthermore, we found correlation between p-MK2-positive tumours with isocitrate dehydrogenase 1 and p53 mutation (Table 1), both markers of lower grade gliomas and secondary GBMs.[35] Thus, MK2 may contribute to the progression of lower grade glioma tumours into GBMs, possibly involving the development of an inflammatory microenvironment enriched with inflammatory cytokines, including IL-6. p-MK2 immunohistochemistry did not correlate with EGFR amplification, a marker of primary, but not lower grade or secondary GBMs.[35] Nevertheless, from a cohort of 44 tumours with EGFR amplification, 18 tumours (41%) expressed activated MK2, suggesting that a subgroup of GBM tumours with EGFR amplification could hinge on the molecular mechanism described in this study.

In summary, this study provides insight into the differential regulation of IL-6 production in the oncogene-microenvironment context of GBM biology. GBM cells expressing oncogenic EGFRvIII are characterised by increased amounts of cytosolic HuR, an mRNA-stabilising protein.[9,20,21] This is associated with upregulated IL-6 secretion, which is a key factor contributing to GBM tumour progression.[3,4] Most importantly, IL-1β-induced and EGFRvIII-exacerbated HuR translocation and IL-6 secretion is efficiently attenuated by p38 MAPK–MK2 pathway inhibition. Mechanistically, EGFRvIII increases nuclear accumulation of p38 MAPK and MK2 in U87 glioma cells. When activated by IL-1β, the proximity and nuclear enrichment of these two inflammatory kinases is associated with enhanced Cdk1 inactivation, HuR dephosphorylation and shuttling, consequently increasing IL-6 mRNA stability and IL-6 secretion (see model in Figure 5). The accumulation of HuR in the cytoplasm of unstimulated EGFRvIII-expressing cells is further connected to upregulation of Cdk1 and CRM1, which is currently under investigation. Most importantly, activated MK2 is expressed in $>50\%$ of investigated GBM tissues, predominantly in lower grade and secondary gliomas, suggesting potential for MK2 inhibitors as anti-inflammatory agents to combat progression of lower grade gliomas into aggressive GBMs.

ABBREVIATIONS

ATF-1, activating transcription factor 1; Cdk1, cyclin-dependent kinase 1; COX-2, cyclooxygenase-2; CREB, cAMP response element binding; CRM1, chromosome region maintenance 1; DTT, dithiothreitol; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; GBM, glioblastoma; HuR, human antigen R; Hsp27, heat-shock protein 27; IDH, isocitrate dehydrogenase; IL, interleukin; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; Mek1/2, MAPK kinase 1/2; MK2, MAPK-activated protein kinase 2; MKK3, MAPK kinase 3; PI3K, phosphatidylinositol-3-kinase; TNF-α, tumour necrosis factor-α.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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