CD47 Activation-induced UHRF1 Over-expression Is Associated with Silencing of Tumor Suppressor Gene *p16*^{INK4A} in Glioblastoma Cells

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Abstract. *CD47, an integrin-associated protein is over-expressed in several tumors including glioblastomas. Activation of CD47 induces proliferation of human astrocytoma cells but not normal astrocytes via an Akt-dependent way. However, the pathways mediating this process are still unknown. The epigenetic integrator UHRF1 (Ubiquitin-like containing PHD and RING Finger 1) is over-expressed in various cancers and plays a vital role in the silencing of numerous tumor suppressor genes including p16*^{INK4A}*, thereby promoting cell proliferation. The aim of the present study was to investigate the role of UHRF1 and p16*^{INK4A} *in CD47-induced effects. Herein we showed that activation of CD47 in human astrocytoma cell lines U87 and CCF- STTG1 (Grade IV), up-regulated the expression of UHRF1 with subsequent down-regulation of p16*^{INK4A}*, thus promoting cell proliferation. Blockage of CD47 using a blocking antibody down-regulated UHRF1 expression, accompanied by a re-expression of p16*^{INK4A}*, conducting to decreased cell proliferation in both cancer cell lines. Neither CD47 activation nor its blocking has any effect on UHRF1/p16*^{INK4A} *expression in normal human astrocytes. Depletion of CD47 in the U87 cell line resulted in down-regulation of UHRF1. We also found that CD47 activated the inflammatory genes IL-6, IL-7 and MCP-1 by a NF-κB-dependent mechanism in human astrocytoma but not in normal astrocytes. In conclusion, the present findings indicate that CD47 activation increases expression of UHRF1 and suggest, for the first time, that CD47 regulates the epigenetic code by targeting UHRF1. This could represent a new pathway towards cell proliferation and metastasis.*

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CD47, also called integrin-associated protein, is a membrane receptor that is involved in several pathophysiological processes including cell proliferation and migration (1-3) (18, 24, 25), infection (23), immune response (42) and apoptosis (15). CD47 expression has been reported to be increased in several hematological and solid tumors (12). CD47 regulates osteoclastogenesis and its disruption causes a decrease in tumor bone metastasis (46). It has been shown that CD47 plays a key role in the progression of breast cancer *via* its interaction with the transmembrane glycoprotein SIRPα (signal regulatory protein α) (33). The CD47-binding peptide 4N1 (RFYVVMWK), derived from the thrombospondin-1 C-terminal domain, was described as inducing cell death (21) and exhibiting antiangiogenic activity (30).

CD47 was found to be highly expressed in multiple myeloma (MM) (40) and human myeloid leukemia stem cells (AML LSC) compared to normal bone marrow and the increase in its level is associated with worse clinical outcome (26). Interestingly, blocking the CD47 function, using the B6H12 monoclonal antibody, selectively promoted phagocytosis of AML LSC by human macrophages (26). In the same context, it has been shown that CD47 receptor expression is increased in acute lymphoblastic leukaemia (ALL) patients compared to their normal cell counterparts and blocking its function was able to eliminate ALL (11). We have previously shown that activation of CD47 induced

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proliferation of human astrocytoma *via* an Akt-dependent manner (43). Altogether these studies indicate that the CD47 receptor is a potential therapeutic target and that its inhibition will lead to blocking pathways involved in the transfer of extracellular signals to the nucleus.

NF-κB (nuclear factor-κB) transcription factor has pro-survival and anti-apoptotic activities on several tumors including glioblastoma (16, 29, 41). Several studies have shown that activation of NF-κB regulates the expression of several anti-apoptotic, pro-metastatic and inflammatory cytokines promoting cell proliferation and survival (27, 34, 35, 39).

UHRF1 (ubiquitin-like containing plant homeodomain (PHD) and really interesting new gene (RING) Finger 1), an oncogene over-expressed in many human cancer cells, encodes an essential protein (UHRF1) required for DNA methylation *via* its affinity for hemi-methylated DNA and its association with DNA methyltransferase 1 (DNMT1) (2, 6, 7, 10). Many studies have shown that UHRF1 plays an important role in G_1/S transition of cell cycle and the epigenetic silencing of various tumor suppressor genes including $p16^{INK4A}$ (2, 4, 13, 19, 20, 31, 47). Interestingly, UHRF1 down-regulation activates tumor suppressor gene re-expression with subsequent cell proliferation inhibition and apoptosis in cancer cells (2, 4, 20, 22).

Considering that UHRF1 negatively regulates tumor suppressor gene $p16^{INK4A}$ expression (2, 47) and has pro-proliferative activities on many cancer cell lines (4), we hypothesized that CD47 decreases tumor suppressor gene expression $p16^{INK4A}$ by increasing UHRF1 expression level, hence cell proliferation enhancement. We also hypothesized that CD47-induced UHRF1/$p16^{INK4A}$ dysregulation is regulated by the NF-κB transcription factor. In the present study we found that CD47 activation induces UHRF1 up-regulation and $p16^{INK4A}$ down-regulation in the human astrocytoma cell lines U87 and CCF-STTG1 (Grade IV) without affecting their expression in normal human astrocytes. Blocking CD47 function allowed re-expression of tumor suppressor gene $p16^{INK4A}$ accompanied by UHRF1 down-regulation in both cancer cell lines. Moreover, a transient knock-down of CD47 in U87 was able to decrease UHRF1 expression. We also found that CD47 regulates the activation of inflammatory genes *IL-6, IL-7* and *MCP-1* by a NF-κB-dependent mechanism in human astrocytoma but not normal astrocytes, suggesting that CD47 receptor up-regulates UHRF1 *via* activation of NF-κB transactivation promoting proliferation of glioma cells. Altogether our results support that CD47-dependent tumor progression involves down-regulation of $p16^{INK4}$ *via* an up-regulation of *UHRF1*.

Materials and Methods

Cell culture and treatment. The human astrocytoma cell line CCF-STTG1 was obtained from the American Type Culture Collection (Rockville, IN, USA), U87 from the European Collection of Cell Cultures (Salisbury, UK) and normal human astrocytes from Lonza (Walkersville, MD, USA). Cell lines were maintained in a humidified incubator with 5% CO_2 at 37˚C and grown in astrocyte basal medium (ABM™) (Lonza, address) for normal human astrocytes, in EMEM for U87, in RPMI for CCF-STTG1 cells. All media were supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 μg/ml gentamycin, 100 U/ml penicillin and 50 mg/ml streptomycin (Sigma, St. Louis, MO, USA). Primary normal human astrocytes were used from the first to the fourth passage. Peptide 4N1 (RFYVVMWK) and the anti-CD47 B6H12 monoclonal antibody (mAb) were purchased from Bachem (Bubendorf, Switzerland) and BD Biosciences (San Diego, CA, USA), respectively.

Cell proliferation assay. Cells were seeded in triplicate on 96-multiwell plates at a density of 5×10^3 cells/well for 24 hours. Cells were treated with different concentrations of the peptide 4N1 or the anti-CD47 B6H12 mAb for 24 h. Cell proliferation rate was then assessed by a colorimetric assay using the Cell Titer 96® AQueous One Solution Cell Proliferation Assay (MTS), following the manufacturer's recommendations (Promega, Charbonnières-les-Bains, France). Absorbance was measured at 490 nm on a multiwell ELISA plate reader.

Western blot analysis. Cells were grown in 6-well plates to 75% confluence and treated with different concentrations of the peptide 4N1 or the anti-CD47 B6H12 mAb for 24 h. Cells were then washed with cold PBS and lysed with ice-cold RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.5) containing protease inhibitors. Equal amounts of total proteins were separated on 10-12% polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes (GE Healthcare, Buckinghamshire, UK), which were then blocked for 1 h at room temperature with 5% nonfat dried milk (BioRad, Hercules, CA, USA). The membranes were then incubated with either a mouse monoclonal anti-UHRF1 antibody (Proteogenix, Schiltigheim, France), a rabbit polyclonal anti-$p16^{INK4A}$ (DeltaBiolabs, Gilroy, CA, USA), a rabbit polyclonal anti-p-IκBα (Merck Millipore, Billerica, MA, USA) or a mouse monoclonal anti-β-actin antibody (Abcam, Paris, France) according to the manufacturer's instructions at 4˚C overnight. The membranes were then washed three times; 5 min/each time with PBS. The membranes were thereafter incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (diluted to 1:10,000 for anti-mouse antibody and 1:5,000 for anti-rabbit antibody) at room temperature for 1 h. The membranes were then washed with PBS five times. Signals were detected by chemiluminescence using an enhanced chemiluminescence kit (GE Healthcare).

Real-time RT–PCR analysis. Cells were grown in 6-well plates to 75% confluence and treated with different concentrations of the peptide 4N1 or the anti-CD47 B6H12 mAb for 5 h. Total RNA was extracted from cells with the PureZOL™ reagent (BioRad) according to manufacturer's recommendations. Reverse transcription was done using 500 ng total RNA with the SuperScript™ III First-strand synthesis system (Invitrogen, Paisley, UK) according to manufacturer's protocol. Amplification was assessed using 1 μL RT products in a mixture containing 200 μM of each dNTP, 0.5 μM oligonucleotide primers, 1x Phusion HF buffer, and 0.02 U/l Phusion DNA polymerase (Finnzymes, Espoo, Finland). The results were normalized to those obtained with *GAPDH* mRNA. The sequences of

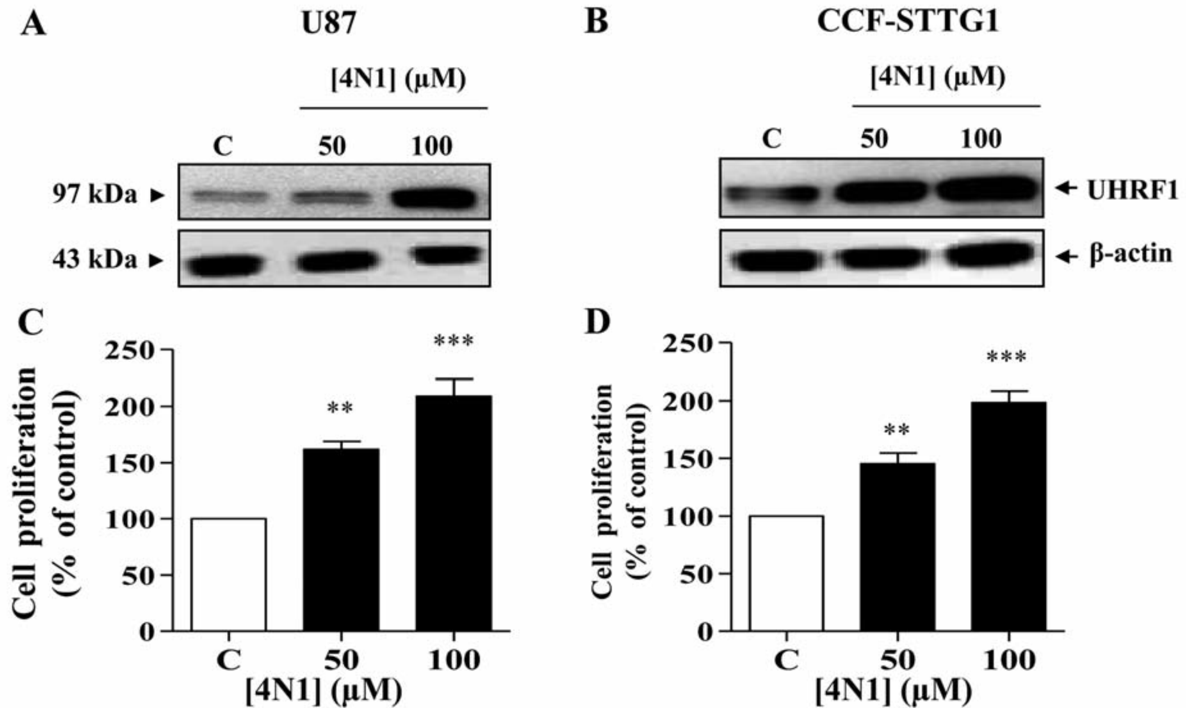

Figure 1. *Effect of CD47-activating peptide 4N1 on UHRF1 expression in human astrocytoma cell lines U87 and CCF-STTG1. U87 cells and CCF-STTG1 cells were incubated with either 50 μM or 100 μM of peptide 4N1 for 24 h (A, B). UHRF1 protein expression was assessed by Western blotting in U87 cells (A) and in CCF-STTG1 (B). Cell proliferation in U87 cells (C) and CCF-STTG1 cells (D) was measured as indicated in the Materials and Methods. Data are shown as mean±SEM of three independent experiments. **p<0.01, ***p<0.001 versus control).*

the primers for PCR amplification were: UHRF1 (sense: 5'-GTCG AATCATCTTCGTGGAC-3'; antisense: 5'-AGTACCACCTCGCT GGCAT-3'); *IL-6* (sense: 5'-TCAATGAGGAGACTTGCCTG-3'; antisense: 5'GATGAGTTGTCATGTCCTGC-3'), *IL-7* (sense: 5'-CTGGGTGAAGCCCAACCA-3'; antisense: 5'- TTCAGTGTTC TTTAGTGCCCATCA-3'), *MCP-1* (sense: 5' - ATG CAATCAATG CCCCAGTC-3'; antisense: 5'-TGCAGATTCT TGGGTTGTGG-3') and *GAPDH* (sense: 5'-GGTGAAGGTCG GAGTCAAC-3', antisense: 5'-AGAGTTAAAAGCAGCCCTGG TG-3'). Cycling parameters were: 95˚C for 30 s, 60˚C for 30 s and 72˚C for 30 s for 30 cycles, followed by a final elongation at 72˚C for 5 min. Amplicons were size-controlled on agarose gel and purity was assessed by analysis of the melting curves at the end of the real-time PCR reaction.

siRNA transfection. U87 cells were transfected with human *CD47* siRNA sequences (On-TARGETplus SMARTpool, Dharmacon RNA Technologies, Lafayette, CO, USA) or non-targeting Pool (Dharmacon, address). Transfections were performed using the INTERFERin™ siRNA transfection reagent (Polyplus transfection, Illkirch, France) following the manufacturer's recommendations.

Statistical analysis. Results are presented as mean±standard error of the mean (SEM) of at least three independent experiments. Statistical analysis was done using analysis of variance (ANOVA). Significant differences are represented throughout as $*p<0.05$, $**p<0.01$, $***p<0.001$.

Results

CD47 is required for UHRF1 expression in astrocytoma cell lines. The effect of the 4N1 (CD47 agonist) peptide and B6H12 (CD47 function-blocking monoclonal antibody) was assessed on UHRF1 expression in human astrocytoma U87 and CCF-STTG1 cells. After 24 h treatment of U87 and CCF-STTG1 cells with peptide 4N1, UHRF1 was increased in a concentration-dependent manner in both cell lines (Figure 1A and B). In order to determine whether CD47-induced UHRF1 up-regulation results from enhanced transcription, *UHRF1* mRNA was quantified in U87 and CCF-STTG1. A significant increase in *UHRF1* mRNA was found in both glioma cell lines starting from 50 μM and with a two-fold increase obtained at 100 μM (data not shown). Proliferation of U87 and CCF-STTG1 cells was analyzed after 24 h treatment with the peptide 4N1. We found that astrocytoma cell proliferation was increased in a concentration-dependent manner in both cell lines (Figure 1C and D). An approximate two-fold increase in proliferation was reached at 100 μM of peptide 4N1. Given that blocking of CD47 function by B6H12 decreases cell proliferation (43) and UHRF1 down-regulation prevents cell proliferation (47), we sought to determine whether blocking

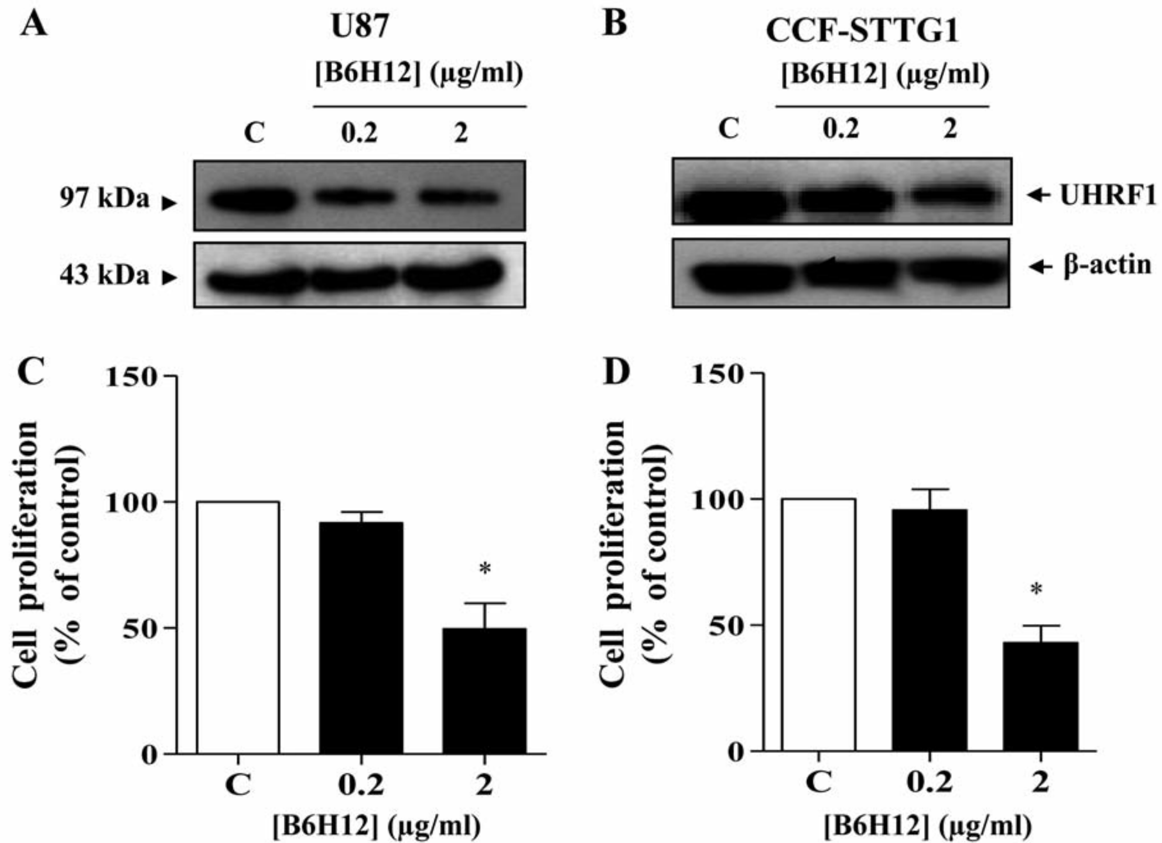

Figure 2. *Effect of CD47 function-blocking mAb (B6H12) on UHRF1 expression in human astrocytoma cell lines U87 and CCF-STTG1. U87 cells and CCF-STTG1 cells were incubated with either 0.2 or 2 µg/ml of anti-CD47 mAb B6H12 for 24 h. UHRF1 protein expression was assessed by western blotting in U87 cells (A) and in CCF-STTG1 (B). Cell proliferation in U87 cells (C) and CCF-STTG1 cells (D) was measured as indicated in the Materials and Methods. Results are shown as mean±SEM of three independent experiments (*p<0.05).*

of CD47 receptor function leads to UHRF1 down-regulation. Figure 2A and B shows that antagonising CD47 function, by using B6H12 antibody, induced a concentration-dependent down-regulation of UHRF1 expression in both human astrocytoma cell lines. This effect was already observed at 0.2 µg/ml and was more pronounced at 2 µg/ml. The decrease in UHRF1 expression was associated with a decreased cell proliferation in U87 and CCF-STTG1. As shown in Figure 2C and D, cell proliferation was significantly inhibited by 50% in both cell lines at 2 µg/ml of B6H12.

CD47 correlates inversely with tumor suppressor gene $p16^{INK4A}$ expression in glioma cells. We and others have shown that UHRF1 down-regulation inhibits cancer cell growth through $p16^{INK4A}$ up-regulation, a downstream target of UHRF1 (2, 47). Therefore, experiments were performed to evaluate whether $p16^{INK4A}$ is regulated by CD47. After a 24 h treatment period of U87 with peptide 4N1, $p16^{INK4A}$ protein levels decreased in a concentration-dependent manner (Figure 3A). Similarly, $p16^{INK4A}$ expression was also decreased in CCF-STTG1 in a concentration-dependent manner (Figure 3B). This down-regulation was significant (Figure 3C and D). Interestingly, treating both cell lines with CD47 function blocking antibody B6H12 at 0.2 µg/ml induced a two-fold $p16^{INK4}$ up-regulation (Figure 3C and D). Taken together, these data suggest that CD47 negatively regulates the cell cycle inhibitor $p16^{INK4}$ with the help of UHRF1, thus promoting glioma cell growth.

To investigate whether basal activity of CD47 is dependent upon UHRF1 expression, we knocked-down CD47 in U87 for 3 days to achieve maximal silencing (up to 90%, Figure 4). Transient knock-down of CD47 (one transfection) resulted in significant depletion of its expression up to 90% in U87 (Figure 4). Interestingly, in CD47-depleted U87 cells, we observed significant down-regulation of UHRF1 (Figure 4). These findings indicate a key role of CD47 receptor in the regulation of UHRF1 expression and, consequently, in cell growth and survival.

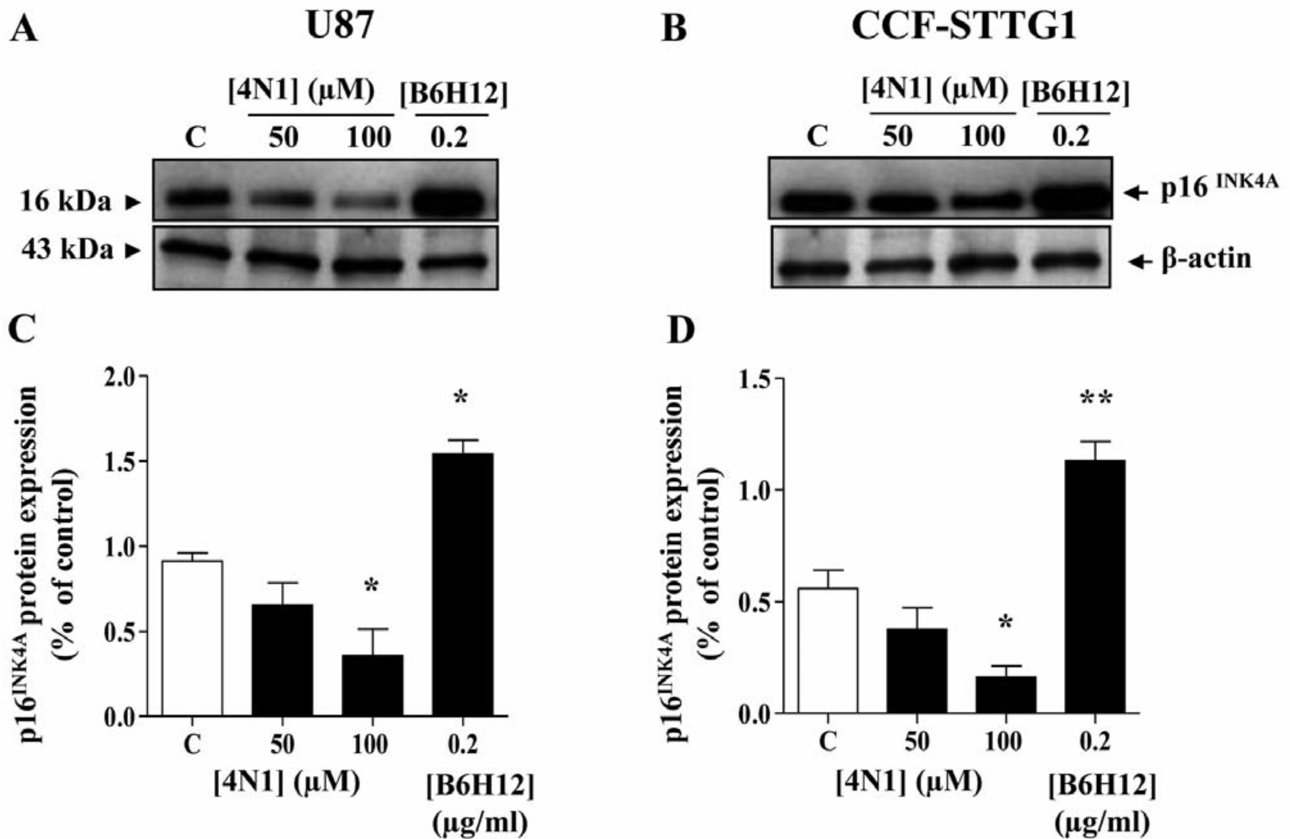

Figure 3. *Effect of CD47-activating peptide 4N1 and blocking mAb (B6H12) on $p16^{INK4A}$ expression in U87 and CCF-STTG1. U87 cells (A) and CCF-STTG1 cells (B) were incubated with either 50 µM or 100 µM of peptide 4N1 or 0.2 µg/ml of anti-CD47 mAb B6H12 for 24 h. $p16^{INK4A}$ protein expression was assessed by western blotting in U87 cells (A, upper panel) and in CCF-STTG1 (B, upper panel). $p16^{INK4A}$ expression levels were analyzed by densitometry and represented as a percentage compared to control (A, B lower panel). Results are shown as mean±SEM of three independent experiments (*p<0.05, **p<0.01 versus control).*

CD47 does not affect UHRF1 and $p16^{INK4}$ expression in normal human astrocytes. Since the activation of CD47 induces proliferation of astrocytoma cells but not of normal human astrocytes (NHA) (43), we investigated whether UHRF1/$p16^{INK4A}$ expression could play a role in the CD47-induced selective proliferation of glioma cells. Interestingly, we observed that activation of CD47 by peptide 4N1 (Figure 5A) or blocking its function using B6H12 (Figure 5B) did affect neither UHRF1 nor $p16^{INK4A}$ protein level in normal astrocytes. Quantification of the bands showed no significant variations (Figure 5 C and D). These findings indicate that activation of CD47 enhances proliferation of astrocytoma cells but not of normal astrocytes probably by regulating UHRF1 and $p16^{INK4A}$ expression.

CD47 regulates NF-κB transactivation and the inflammatory genes IL-6, IL-7 and MCP-1 in glioma cells. Previous studies have shown that the NF-κB transcription factor has pro-survival and proliferative activities in several cancer cells

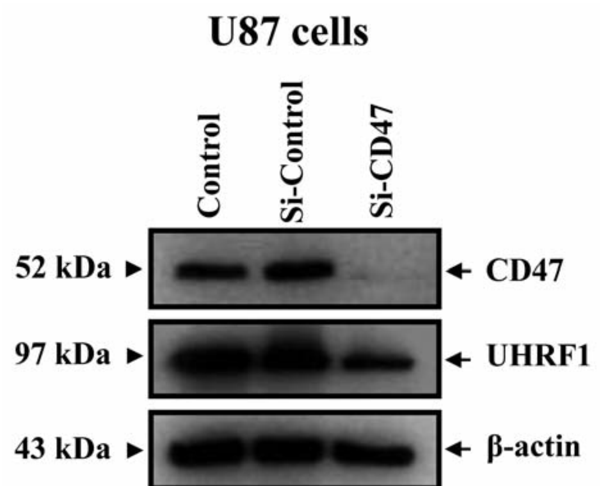

Figure 4. *Effect of down-regulating CD47 on UHRF1 expression. U87 cells were treated with siRNA against CD47 for 3 days to test the silencing effect of CD47 gene on the expression of UHRF1.*

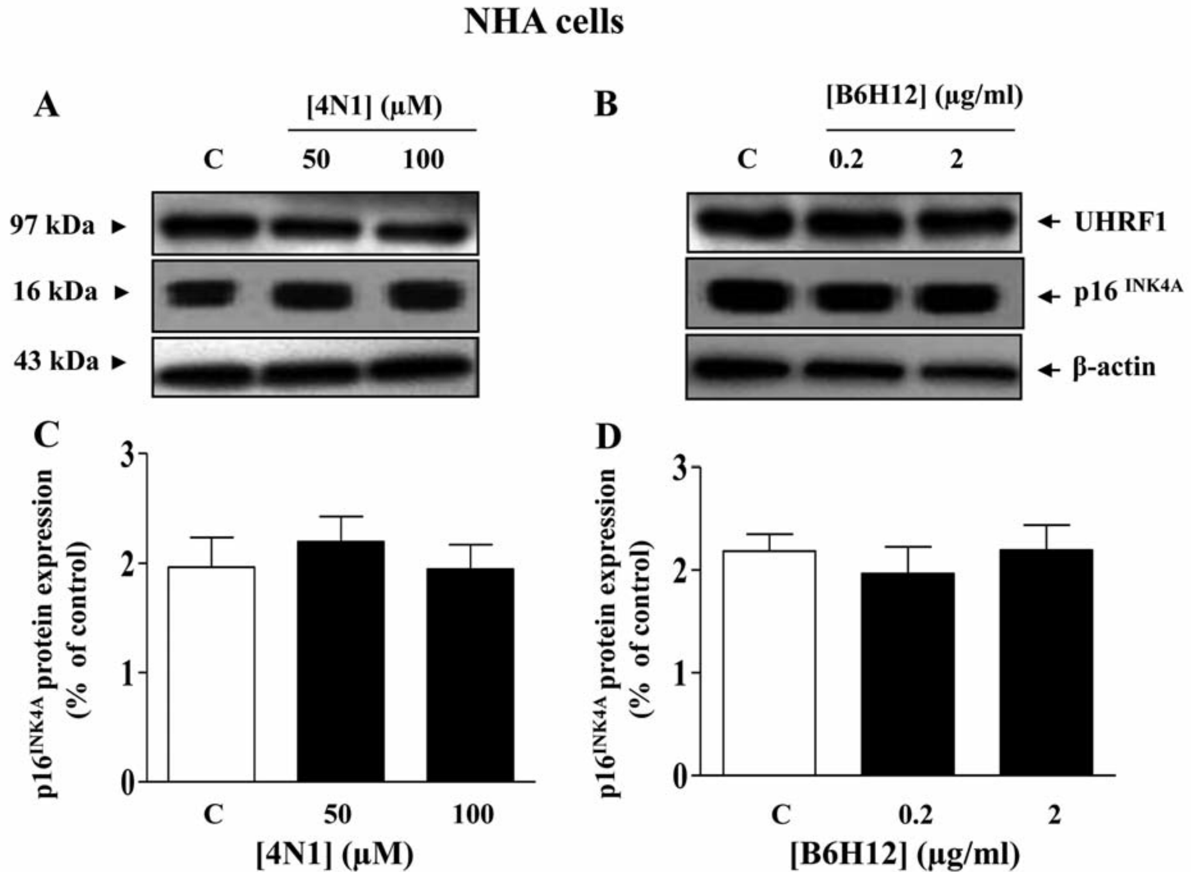

Figure 5. *Effect of CD47-activating peptide 4N1 and blocking mAb (B6H12) on UHRF1 and p16^{INK4A} expression in normal human astrocyte cell line NHA. The human astrocyte cell line NHA was incubated with either 50 μM or 100 μM of peptide 4N1 (A) and either 0.2 or 2 μg/ml of anti-CD47 mAb B6H12 (B) for 24 h. UHRF1 and p16^{INK4A} protein expression levels were assessed by western blotting (A, B upper panel). UHRF1 and p16^{INK4A} expression levels were analyzed by densitometry and represented as percentage compared to control (middle and lower panels, respectively). Results are shown as mean±SEM of three independent experiments.*

including human glioblastoma cells (32). We investigated the effect of CD47 activation on the phosphorylation of IκBα, which is an indicator of NF-κB transactivation. Figure 6A and B indicate that activation of CD47 induced a concentration-dependent increase in p-IκBα expression level in glioma cells. The increase in the level of IκBα phosphorylation was more pronounced in U87 (Figure 6A) than in CCF- STTG1 (Figure 6B). A slight effect on p-IκBα was observed in NHA (Figure 6C). Conversely, blocking of CD47 function by B6H12 mAb decreased the phosphorylation level of IκBα (Figure 6 A and B) in both cell lines without affecting its level in NHA (Figure 6C). Then, we analyzed the expression of NF-κB-regulated inflammatory genes *IL-6, IL-7* and *MCP-1* in both cell lines and in NHA. Treating astrocytoma cells with peptide 4N1 increased the expression of *IL-6, IL-7* and *MCP-1* mRNA in a concentration-dependent manner (Figure 6A, B, lower panel). Unlike in astrocytoma, treating NHA with the same concentrations of 4N1 had no effect on cytokine expression level (Figure 6C, lower panel). Interestingly, blocking CD47 function induced a concentration-dependent decrease in the level of *IL-6, IL-7* and *MCP-1* mRNA in both astrocytoma cell lines grade IV (Figure 6A, B, lower panel), while there was no effect on their expression in NHA (Figure 6C, lower panel). Altogether, these results indicate that CD47 selectively activates NF-κB transactivation likely by phosphorylation of its inhibitor IκBα leading to the expression of IL-6, IL-7 and MCP-1 in astrocytoma.

Discussion

The role of CD47 in promoting cell growth and enabling tumors to escape the immune response has been studied extensively but the pathways involved in this process still remain elusive. Our findings show that CD47 activation induced an up-regulation of the anti- apoptotic protein UHRF1 associated with a down-regulation of the tumor suppressor

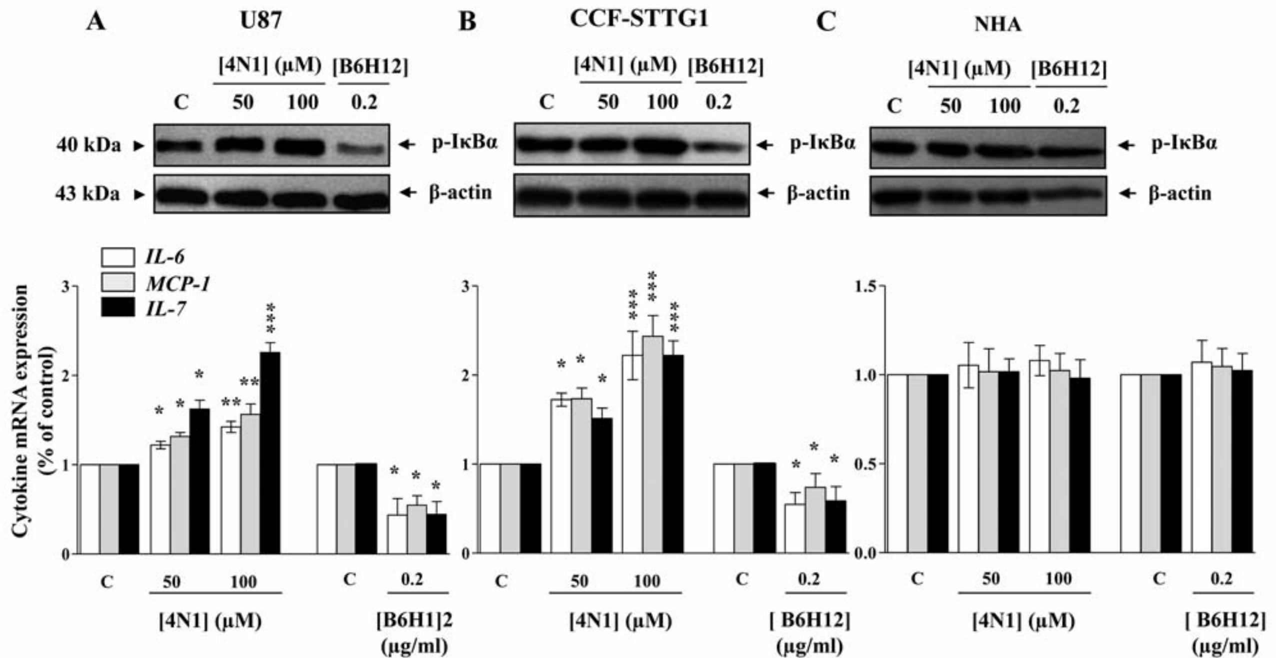

Figure 6. *Effect of CD47-activating peptide 4N1 and blocking mAb (B6H12) on p-IκBα, IL-6, IL-7 and MCP-1 expression in U87, CCF-STTG1 and in NHA. U87 cells (A), CCF- STTG1 cells (B) and NHA (C) were incubated with either 50, 100 μM of 4N1 peptide or with 0.2 μg/ml of anti-CD47 mAb B6H12 for 24 h and for p-IκBα and for 5 h. p-IκBα expression was assessed by western blotting (A, B, C). mRNA levels were evaluated by RT-PCR in peptide 4N1-treated U87 (A, lower panel), CCF-STTG1 cells (B, lower panel) and in NHA (C, lower panel). Data are shown as mean±SEM of three experiments (*p<0.05, (**p<0.01, **p<0.001 versus control).*

gene $p16^{INK4A}$ in human astrocytoma cells without affecting their expression levels in human normal astrocytes. Moreover, blocking of CD47 function induces UHRF1 down-regulation associated with $p16^{INK4A}$ up-regulation in both astrocytoma cell lines. To our knowledge this is the first report to show an up-regulation of UHRF1 following a physiological ligand-receptor interaction. Conversely, we have previously shown that TCR activation induced down-regulation of UHRF1, after which we proposed that UHRF1 might act as an anti-apoptotic factor (1). In the present study, we proposed that CD47 activation needs the help of a UHRF1 increase to inhibit the activation of pro-apoptotic pathways and, thus, to promote cell proliferation.

One apoptotic pathway that might be targeted by UHRF1 is the one involving the tumor suppressor gene $p16^{INK4A}$. Indeed, we have previously shown that UHRF1 down-regulation induces an activation of the tumor suppressor gene $p16^{INK4A}$ in Jurkat cells (2). Recently, it has been shown that UHRF1 over-expression in colorectal cancer cells is associated with silencing of tumor suppressor gene $p16^{INK4A}$ and tumoral progression and that UHRF1 down-regulation induced a significant decrease in proliferation and migration of colorectal cancer cells associated with $p16^{INK4A}$ up-regulation (47). Our study showed that blocking CD47 function induces UHRF1 down-regulation and $p16^{INK4A}$ up-regulation in astrocytoma cell lines but not in normal cells, although they have similar CD47 levels (43). Therefore, the increased UHRF1 expression, accompanied by a decrease of $p16^{INK4A}$ expression as an ubiquitinous mechanism of tumorogenesis, is also taken by CD47.

We have shown previously that activation of CD47 induced a rapid phosphorylation of Akt, a downstream target of PI3-kinase in human astrocytomas but not in human normal astrocytes (43). Signaling through the PI3-kinase is commonly activated in many tumors including glioblastoma and leads to activation of the pro-survival NF-κB pathway (37). The present findings showed in U87 and CCF-STTG1 that activation of CD47 by its agonist 4N1 stimulates the activation of NF-κB transcription factor likely by inducing phosphorylation of its inhibitor IκBα with the subsequent up-regulation of several inflammatory genes including *IL-6, IL-7* and *MCP-1*. Activation of the transcription factor NF-κB is observed in several tumors including glioblastoma and stimulates the expression of various oncogenes and factors involved in inflammation, cell cycle, apoptosis and angiogenesis (3, 8, 9, 14, 16, 28, 29, 36, 44, 48). NF-κB activation has been shown to inhibit the tumor suppressor gene *p53* in glioblastoma (grade IV) (17, 38, 45). Considering that the tumor suppressor gene

p53 negatively regulates UHRF1 expression (5), we suggest that CD47-mediated NF-κB activation leads to UHRF1 over-expression inhibition of the p53 pathway.

Conclusion

The present findings indicate that the selective proliferative effect of CD47 in glioblastoma cells involves up-regulation of UHRF1 and down-regulation of the tumor suppressor gene $p16^{INK4A}$, which could be mediated by a NF-κB-dependent pathway.

Furthermore, the present findings indicate that CD47-induced NF-κB activation up-regulates several inflammatory genes promoting growth and metastasis of astrocytoma cells. This study supports the concept that CD47 is a potential target for anticancer therapy and that blockage of its function helps inhibit cancer cell proliferation most likely by decreasing UHRF1 and increasing $p16^{INK4A}$ expression.

Conflicts of Interest

The Authors declare that they have no competing interests.

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