The constitutively active HCMV-encoded receptor UL33 displays oncogenic potential

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Manuscript in preparation
Abstract

The human cytomegalovirus (HCMV), associated with the development of malignancies, encodes the constitutively active G protein coupled receptors (GPCRs) UL33 and US28. As US28 possesses oncogenic properties and is expressed in CMV positive human glioblastomas, we set out to investigate the signaling properties of UL33. US28 expression is detected early after infection, whereas UL33 is expressed at later stages. In transient, as well as stable transfection systems, UL33, like US28, constitutively activates various proliferative, pro-angiogenic signal transduction pathways. In xenograft models, UL33 was shown to induce tumor growth. As shown previously for US28, UL33 expression was also detected in human glioblastoma. Interestingly, the distribution of UL33 in the primary glioblastoma samples was more dispersed, while expression of US28 was confined to the vascular niche. Taken together, our data indicate that the viral GPCR UL33 has oncogenic potential and could play a role in HCMV-associated malignancies. The differential kinetics of UL33 and US28 expression and pronounced expression of UL33 in human glioblastoma, indicate that HCMV has devised distinct means to engage or prolong proliferative signaling pathways upon infection through expression of these viral receptors.
4.1 Introduction

The mammalian line of defense against invasion of pathogens or injury depends on timely activation and proper targeting of leukocytes [254, 255]. Chemokine-chemokine receptor interactions are crucial to control both onset and localization of leukocyte-mediated responses. Several β-herpesviruses have corrupted this system by hijacking and subverting components, both ligands and receptors, of the chemokine system [80, 129, 256]. Human cytomegalovirus (HCMV, HHV5), a member of the β-herpesvirus subfamily has successfully utilized this strategy resulting in high prevalence with up to 90% of the population harbouring a latent infection [129]. While HCMV infection is asymptomatic in immune-competent individuals, it can cause severe pathologies in immune-compromised patients [3, 257]. Moreover, HCMV infection during pregnancy is the leading infectious agent causing mental retardation and deafness of the unborn child [2]. Furthermore, HCMV infection is associated with several pathologies, such as atherosclerosis [133], autoimmune diseases and cancers [3, 257-259]. HCMV infection has been proposed to aggravate the malignant phenotype of e.g. colon cancer [257] malignant glioma [3, 260] and medulloblastoma [234]. The HCMV-encoded chemokine receptor US28 is one of the HCMV-encoded proteins believed to contribute to the malignant phenotype [80, 161, 234].

US28 has been shown to scavenge and internalize different members of the CC family chemokines including CCL5 and CCL2, and also CX3CL1 [155]. Furthermore, US28 has been shown to constitutively stimulate proliferative signaling [6, 80-82]. Moreover, this GPCR have been implicated in oncomulation in vivo, as heterologous expression of US28 in mice, stimulates intestinal neoplasia [87]. Furthermore, expression of US28 was correlated to proliferative signaling and poor prognosis in human glioblastoma [80].

Besides US28, HCMV encodes three other viral G protein coupled receptors (vGPCRs) with homology to human chemokine receptors; US7, UL33, and UL78 [256]. These receptors and two chemokine homologues (vCXCL1 and 2) [144] may be utilized by the virus to deceive the host’s immune system and could be instrumental in viral pathology. Homologues of UL33 and UL78 are conserved throughout the β-herpesvirus family [262]. In contrast, US28 and US27 have been identified only in CMVs, closely related to HCMV, targeting primate hosts [263].

Like US28, UL33 has been shown to signal in a constitutive manner via Gaq, Gai, and Gao [156]. The constitutive activation of both receptors could be instrumental in HCMV-mediated pathology. Therefore, it is crucial to determine the kinetics and cellular consequences of signaling mediated by these vGPCRs. The
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UL33 homologue in the murine cytomegalovirus (M33) was shown to be functional in viral dissemination [88]. Interestingly, loss of M33 can be partially complemented with functional UL33, suggesting conservation of functionality. Both UL33 and US28 were found to co-localize with the viral envelop glycoproteins gB and gH [145, 146]. Although UL33 [145] and US28 [147] are dispensable for viral replication in respectively MCR5 and human foreskin fibroblasts, functional consequence of expressing vGPCRs on the viral particle may enhance binding of the virus particles to target cells. Alternatively, these receptors could exert their effect by rewiring cellular signaling of the host cells.

In this study we describe the UL33-mediated constitutive activation of proliferative signal transduction pathways, and consequences for cellular transformation. Moreover, we detected the expression of this vGPCR in human glioblastoma. As UL33 and US28 expression patterns after HCMV infection revealed different kinetics, our data suggest HCMV may use both UL33 and US28 to successfully engage proliferative signaling pathways in the host cell.

4.2 Results

UL33 is expressed with different kinetics compared to US28 in HCMV infection cycle

As both US28 and UL33 were previously shown to constitutively signal via multiple Gα-dependent signaling pathways, we examined the possibility of these events to occur in concert by monitoring expression of both receptors during the HCMV infection cycle. We evaluated the kinetics of US28 and UL33 expression after infection of human foreskin fibroblasts (HFF) using the HCMV TB40-BAC4 strain. We analyzed the kinetics of UL33 expression taking advantage of the N-terminally Flag tagged UL33 (TB40-BAC4-UL33ex1-FLAG). US28 was visualized using the antibody described [80]. Intracellular distribution of both US28 and UL33 was similar as described earlier [146, 264] with UL33 mostly present in intracellular vesicles and US28 concentrated in the perinuclear area (data not shown). The kinetics of expression of both UL33 and US28 were investigated in situ and by Western analysis (Figure 4.1A and B). As can be seen in Figure 4.1A, expression
Figure 4.1: Kinetics of expression UL33 and US28 after HCMV infection. (A) UL33 receptor expression after infection with TB40 UL33ex1-FLAG (green) increases 96, 120, and 144 hours post infection in HFF cells. US28 (red) is expressed 24 hours post infection, with strongest expression from 48 hours post infection and onwards. All micrographs have been taken at 40x magnification. Nuclei of the cells were stained with DAPI (blue). (B) Western-blot analysis of both UL33ex1-FLAG and US28 expression in infected cells. The US28 antibody typically stains two bands of approximately 45 kD and 40 kD as shown in the panel. Expression of Flag-tagged UL33 (35 kDa) is observed at 96, 120 and 144 hours post infection (h.p.i.), whereas US28 is already detectable at low levels 24 h.p.i. Presence of US28 increases at later time-points. (C) [125I]-CCL5 binding shows early kinetics of US28 protein expression in AD169-WT-infected cells with a peak at 48 h.p.i. No [125I]-CCL5 binding is detected in cells infected with AD169-ΔUS28 mutant or in UV-inactivated WT virus infected cells. (D) PLC activation is significantly reduced in the absence of US28 in TB40-BAC4 deletion mutant virus infected cells 48 h.p.i. Inositol phosphate accumulation performed on HFF cells, infected with m.o.i. of 2 of different deletion HCMV TB40-BAC4 strains shows significant reduction in inositol phosphate accumulation by US28 and a double deletion mutant viral strain compared to wild-type infected cells (*p < 0.05, **p < 0.01, ***p < 0.001 respectively). Virus m.o.i. used in infections was confirmed to be comparable by back titration (data not shown).
of UL33ex1-FLAG was observed at 96, 120 and 144 hours post infection (h.p.i.), whereas US28 is already detectable 24 h.p.i., at low levels as indicated by Western blotting (Figure 4.1B). This may indicate an exclusive role of US28 early after infection and collective effects of both receptors, later in the infection cycle. Confirmation of US28 expression early in the HCMV infection cycle was obtained by means of radioligand binding assays, using [125I]-CCL5, a chemokine known to bind US28. In these analyses, increased binding of [125I]-CCL5 was observed 48 hrs after infection with the wild type HCMV strain (Wt) but not with the ΔUS28 (US28 deletion strain) - or UV-inactivated virus (Figure 4.1C). As no ligands for UL33 have been identified to date, these assays could not be performed for this vGPCR. To examine the functional consequence of differential expression of both viral GPCRs, PLC activation was measured in HCMV infected cells. For this purpose, TB40 wild type and mutant viral strains, deleted for UL33, US28 or both were used [265]. All HCMV mutants used in this study displayed similar infection properties as confirmed by back titration (data not shown). Activation of PLC in HFF was significantly reduced for ΔUS28 and the double deletion mutant ΔUS28-ΔUL33 viral strains 48 h.p.i. (Figure 4.1D). Deletion of UL33 alone did not result in a significant reduction of PLC activation 48 h.p.i.

**HCMV-encoded UL33 constitutively activates multiple signaling pathways**

As US28 was described to promiscuously trigger activation of various proliferative-, angiogenic- and inflammatory signaling pathways resulting in oncomodulatory effects [80–82], we evaluated UL33-mediated activation of these signaling pathways. As can be seen in Figure 4.2A, transfection of increasing amounts of pcDEF3-UL33 increased levels of UL33 protein as detected in an ELISA using anti-UL33 antibody [145]. UL33 constitutively induced VEGF promotor activity in a dose-dependent manner, as was measured with a specific reporter gene (Figure 4.2B). Likewise, UL33 expression induced activation of the NFAT pathway, COX-2 promotor activity and STAT3 transcriptional activity as measured with specific reporter genes when expressed in HEK293T cells (Figure 4.2C-E).
Figure 4.2: Viral chemokine receptor UL33 constitutively activates various signal transduction pathways. (A) ELISA of expression of UL33 after transfection with various amounts of the pcDEF3-UL33 to HEK293 cells. Bars represent the expression in permeabilized cells. Anti-UL33, kindly provided by Prof. W. Gibson, was used to detect UL33. (B) UL33 dose-dependently activates VEGF promoter activity. UL33 also induces NFAT transcriptional activation, (C) COX-2 promoter activation and (D) STAT3 transcriptional activation. (E) For all reporter gene assays HEK293T cells ($1 \times 10^6$) are transiently transfected with 25 ng UL33 DNA in combination with the respective reporter constructs. Luciferase expression was determined 24 hrs post transfection. Results are presented in relative light units (RLU) and are all normalized (Mock is 100).
UL33 induces a transformed phenotype in NIH-3T3 cells

To investigate whether UL33 expression was associated with onset of transformation, as shown earlier for US28 [6], stable NIH-3T3 cell lines expressing UL33 were generated. To this end, wildtype UL33 and C-terminally eGFP-tagged UL33 constructs were used. Constitutive PLC activation indicated proper receptor functionality and expected signaling properties of both wt UL33 and eGFP-tagged UL33 receptors (Figure 4.3A). The observed increase in PLC activation in cell lines expressing UL33 was lower compared to those in US28-expressing NIH-3T3 cells.

To examine the proliferative potential of UL33, a thymidine incorporation assay was done. Clearly, cell lines expressing UL33 displayed increased [3H]-thymidine incorporation. DNA synthesis upon serum starvation was two-to four-fold higher in UL33-expressing cells compared to mock transfected cells. To confirm the oncogenic potential of UL33 a foci formation assay was performed. As depicted in Figure 4.3C UL33-eGFP expressing cells lost contact inhibition and formed foci as seen for US28-expressing cells, albeit with a lower efficiency compared to US28. In view of convergence of UL33 signaling into the STAT3 pathway, observed earlier for US28 [80], cells were treated with stattic, a small inhibitor molecule inhibitor targeting STAT3 [266]. Stattic inhibited foci formation in UL33-expressing cells to a similar extent as seen in US28-expressing cells (Figure 4.3D). Based on the enhanced increase in PLC activation, thymidine incorporation and foci formation, the stable UL33-eGFP expressing 3T3 clone #4 was selected for further analyses in vivo.

UL33 stimulates proliferative signaling

Since in vitro studies with UL33-expressing cells showed a transformed and proliferative phenotype, we determined whether UL33-eGFP expressing cells could also induce tumor formation in vivo. To this end, UL33-eGFP or US28-expressing NIH-3T3 cells were subcutaneously (s.c.) injected in the flanks of nude athymic mice (6 mice injected per cell line; 12 inoculations). First signs of tumor formation appeared as early as 1 week post injection for the mice injected with US28-expressing cells, and shortly after that for the mice injected with the UL33-eGFP cells, as shown in the Kaplan-Meier curve (Figure 4.4B). Tumors were clearly visible 2-3 weeks after inoculation (Figure 4.4A), after which animals were sacrificed. Some mice injected with UL33-expressing cells displayed smaller tumors at the exterior, however, large internal tumors were present in all cases. The mock group,
Figure 4.3: UL33 induces a proliferative phenotype in NIH-3T3. (A) UL33 shows constitutive inositol phosphate accumulation in NIH-3T3 cells stably expressing UL33 wt and UL33-eGFP. Constitutive accumulation of inositol phosphate in NIH-3T3 cells expressing US28 was used as positive control. (B) UL33-expressing NIH-3T3 cells display increased proliferation as measured by $[^3]$H-thymidine incorporation. Data are normalized over 3T3-Mock values. $[^3]$H-thymidine incorporation by NIH-3T3 cells stably expressing US28 was used as positive control. (C) NIH-3T3 cells expressing UL33-eGFP show loss of contact inhibition as evidenced by the appearance of foci, albeit to a lesser degree compared to NIH-3T3 cells expressing US28. (D) Inhibition of foci formation by NIH-3T3 cells stably expressing UL33-eGFP and US28, respectively, in the presence of 1 µM Stattic (specific inhibitor of STAT3 dimerization).
as previously published [6], did not develop tumors up to 75 days after injection. Gene expression of US28 and UL33 was confirmed by RT-PCR in the tumors formed (Figure 4.4C). For US28 a much higher expression (>256x higher) was obtained than for UL33 mRNA.

UL33 expression in primary glioblastoma

Previous study from our laboratory has shown that US28 is expressed in primary glioblastoma tumors [80]. To examine the potential presence of UL33 in primary glioblastoma, we examined tissue samples of 25 glioblastoma patients by immunohistochemistry using antibodies raised against UL33. UL33 was expressed at different levels in GBM tumors, mainly in the tumor cells that appear as patches in the tissue (Figure 4.5 B –D and F). UL33 staining is primarily cytoplasmic (Figure 4.5D). Patients were categorized in two groups (based on approximately percentage number of UL33 positive cells in their tumors (negative or grade1; <10% and grade 2; >10%-50%). UL33 was expressed in <10% of the tumor cells in 15/25 (60%), in >10-50% in 10/25 (40%). One of the samples tested in this study was negative for UL33. Median overall survival was 15.5 months in group 1 vs 13 months in group 2 of patients (p= 0.09, Figure 4.5G). Time to first tumor progression was 5 months longer in group 1 patients compared to group 2 patients.
(13 vs 8 months, $p=0.67$, Figure 4.5H). 5 patients were alive; 3 patients in group I and 2 patients in group 2.

Expression of UL33 around vessels, the main site for expression of US28 in primary glioblastoma, in these tissue samples (Figure 4.5F) was detected in 9 out of the 25 (36%) of tumor samples. In these cases mostly endothelial cells and smooth muscle cells (SMC) appeared to be positive for UL33 staining. Interestingly, infiltrating neutrophils and granulocytes appear to be strongly positive for UL33 in 8/25 (32%) of these patients (Figure 4.5F).

4.3 Discussion

Herpesviruses are known to alter cellular gene expression and cell function [176, 267]. While the oncogenic potential of some viruses like Kaposi’s sarcoma associated herpes virus and human papilloma virus is well established [186, 268], HCMV is believed to possess oncomodulatory properties. In view of the oncomodulatory potential of the HCMV-encoded chemokine receptor US28 [6, 80, 87, 161, 234], we subjected UL33, another constitutively active viral encoded chemokine receptor homolog [156] to signaling and cell proliferation studies. In this study we clearly demonstrate that UL33, like US28, has oncogenic potential in vitro as well as in vivo. UL33 constitutively activates several signaling pathways (Figure 4.2), which play key roles in enhanced cellular proliferation and oncogenesis [202, 269, 270]. UL33 stimulated transcriptional activation of NFAT and STAT3 (Figure 4.2C and 4.2E) and induced VEGF and COX-2 promoter activation (Figure 4.2B and 4.2D). NIH-3T3 cells stably expressing UL33 exhibit constitutive PLC activation, increases in DNA synthesis rate and loss of contact inhibition (Figure 4.3), properties indicative of transformation. UL33-eGFP-expressing NIH-3T3 cells induce tumor growth within 3 weeks in a nude mice xenograft model (Figure 4.4). Tumor formation was apparent after 2 weeks, while did occur earlier for US28. The slower onset of tumor formation compared to mice injected with US28-expressing NIH-3T3 cells, can be explained by reduced increases in PLC activity and foci formation UL33 compared to US28-expressing NIH-3T3 cells. Moreover, the UL33 mRNA levels in the xenografts are significantly lower compared to US28 mRNA levels in US28-induced xenografts (Figure 4.5C), suggesting that the oncomodulatory effects of both US28 and UL33 may differ, despite the overlap in receptor-induced
signaling we have observed. Noteworthy, unlike for US28 [80], increased IL6 levels could not be detected in medium from NIH-3T3 cells stably expressing UL33 (data not shown) nor could we measure increased PGE2 levels. Although both vGPCRs can activate a comparable set of signaling pathways, a certain threshold of activation may be required to induce rapid onset of tumorigenesis.

To study the expression kinetics of UL33 and US28 in HCMV infected cells, human foreskin fibroblasts (HFF) were infected with a mutant HCMV virus (BAC40-UL33ex1-Flag). Intracellular localization of both US28 and UL33 was comparable as described earlier [146]. The kinetics of expression of both vGPCRs differ, with US28 being detectable 24 h.p.i., whereas UL33 only appeared after 72 h.p.i. Early expression kinetics of US28 was confirmed in a CCL5 binding assay and by measuring PLC activation 48 h.p.i. using HCMV TB40 WT and mutant strains lacking either US28, UL33 or both vGPCRs [265]. Our data show that UL33, unlike US28, is expressed at a late stage of the HCMV replication cycle. This confirmed earlier observations on US28 and UL33 expression profiles in HFF and U373-MG cells [146, 147, 271]. In a viral setting, both receptors could have modulatory functionalities important in HCMV pathology. As the UL33 expression kinetics is slower compared to that of US28, this may result in further activation of signaling pathways initially triggered by US28. For instance, the enhanced COX-2 promoter activity as previously described for US28 [81], could be prolonged by UL33 late expression. A comparable synergism could be postulated for the activation of STAT3 signaling important in US28 mediated oncomodulation [80]. Yet, co-expression of both receptors could also result in antagonizing effects. Recently, UL33 was reported to co-localize and form heterodimers with US28 [89]. Interestingly, the constitutive activation of the US28-mediated Gαq/phospholipase C pathway was not affected by receptor heteromerization, while UL33 was able to silence US28-mediated activation of the transcription factor NF-κB. These data imply that co-expression may result in inhibitory effects on downstream signaling. Further experiments are required to substantiate whether co-expression of both receptors does result in altered proliferative signaling.

Besides temporal synergy or modulatory function between the vGPCRs also a spatial complementarity between UL33 and US28 could affect HCMV-induced pathology. In this study we have shown that UL33 is expressed at detectable levels in primary glioblastomas (Figure 4.5). Compared to our earlier observation regarding expression of US28 in glioblastoma the pattern of UL33 expressed in these tumors is more pronounced. Whereas US28 is primarily expressed around the blood vessels within the so-called vascular niche [80], UL33 expression is detected in the vessel area as well within the cells that make up the tumor tissue
Figure 4.5: UL33 expression in primary glioblastoma specimens. Representative immunohistochemical stainings are shown. (A) and (E). Rabbit IgG isotype control staining (B), (C), (D) and (F). Samples stained with anti-UL33 antiserum (brown). Scale bars 100 μm. (F) Patient overall survival (OS) and (G) time to tumor progression (TTP) are not significantly affected by UL33 expression.
and in immune cells invading the tissue. However, UL33 expression levels do not significantly correlate with patient outcome and survival.

Taken together, our data indicate that the viral GPCR UL33 has oncogenic potential and could play a role in HCMV-associated malignancies. The differential kinetics of UL33 and US28 expression and pronounced expression of UL33 in human glioblastoma, indicate that HCMV has devised distinct means to engage or prolong proliferative signaling pathways upon infection through expression of these viral receptors.

### 4.4 Materials and Methods

**Cell Culture.** HEK293T cells, human glioblastoma U373 cells, and mouse fibroblast NIH-3T3 cells were cultured in DMEM supplemented with penicillin/streptomycin (50 IU/ml) and 10% of fetal bovine, heat inactivated fetal bovine or bovine serum, respectively. Transfections were performed in HEK293T with the polyethylenimine (PEI) method [252]. Transfections in U373 and NIH-3T3 cells were performed with the calcium phosphate method. Stably transfected NIH-3T3 cells were selected and maintained in culture with neomycin (400 μg/ml) to ensure expression of UL33 and US28 receptors, respectively.

**Receptor Characterization by InsP formation and Thymidine Incorporation.** UL33 or US28 expression and constitutive signaling were analyzed for inositol phosphate formation as previously described [6]. As for Thymidine incorporation measurement, the experiment was carried out upon serum starvation by using medium containing 0.5% calf serum.

**Reporter Gene Analysis.** For the promoter activation measurements, 10^6 HEK293T cells were transfected with 0.5-1.5 μg of reporter-Luciferase plasmid and the indicated amounts (25 ng or 1 μg) of pcDEF3-UL33. To measure VEGF promoter activation, (500ng/1×10^6 cells) the pGL2-VEGF-LuciferaseLuciferase construct, kindly provided by Dr. G. Pages (Institute of Signaling Development Biology and Cancer, Nice, France) was used. The Ly6E STAT3-response element luciferase construct (500ng/1×10^6 cells) [272] was used for STAT3 activity measurements. The NFAT reporter gene (500ng/1×10^6 cells), pNFAT-luc was purchased from Strata-
gene. Total DNA amounts were kept constant using empty vector pcDEF3. In U373 infected cells, transfection of the VEGF-Luciferase plasmid [6] was performed 2 h post infection (multiplicity of infection 1). Luciferase activities were measured 24 h after transfection with a Victor² multilabel plate reader from Perkin-Elmer.

**Focus Formation Assay.** The focus formation assay was performed as described earlier [273]. 400 stably transfected NIH-3T3 cells were cultured with 2000 untransfected NIH-3T3 cells for 2 weeks in regular culture medium without G418 in 6 well plates. Treatment with Stattic was initiated 12 hours after seeding. Medium containing Stattic was replaced every 48 hours after seeding. After 2 weeks, the cells were washed three times with phosphate buffered saline (PBS) and subsequently fixed in cold methanol for 10 min. Subsequently, the cells were stained with 0.4% methylene blue in H₂O and the foci were counted.

**Tumor Formation in Vivo.** All animal experiments were performed according to the National Institutes of Health principles of laboratory animal care and Dutch national law and approved by the Dierexperimentencommissie from the VU Medical Center and performed in compliance with the protocol FaCh 05-02. Stably transfected NIH-3T3 cells (2 × 10⁶) containing pcDEF3-US28, or pcDEF3-UL33-eGFP plasmids were injected s.c. into the flank of 8- to 10-week-old female nude mice (Hsd, athymic nu/nu, 25–32 g, Harlan Laboratories Cambridge Research Biochemicals; Zeist, The Netherlands).

**RT-PCR.** In the UL33-eGFP- and US28-expressing NIH-3T3 cells as well as the tumors formed, UL33 and US28 gene expression was checked using standard reverse transcriptase PCR (RT-PCR) as previously described [81]. For detection of US28 mRNA the primers used were US28 forward 5-AGCGTGCCCGTGACGACGTTAC-3 and US28 reverse 5-ATAAAGACAAACCGACC-3. For detection of UL33 mRNA the primers used were UL33 forward 5-GGAAAGTGCTGCTGACGCTAG-3 and UL33 reverse 5-GCTGTACGGTTGAGTAGAAGAAGG-3.

**HCMV infections and Western blotting.** U373 MG cells were infected with TB40-BAC4-HCMV [265] strains containing the wild type genotype or a mutant version. Using a recombinant virus encoding a FLAG-tagged UL33 expression and sub cellular localization of UL33 was studied after HCMV-infection. 2 × 10⁵ HFF cells were seeded in 6-well plates and infected the next day at m.o.i. of 2. After 2 hrs, virus was removed and cells were washed 3 times with PBS to enable a synchronized infection. After indicated time (1, 2, 3, 4, 5, 6, 7 or 8 dpi) cells were washed 3 times with cold PBS, scraped and stored as a dry cell pellet at -
Cell lysates were prepared in Ripa buffer containing protein inhibitors, lysates were sonicated for 10 sec. 12 μg of total protein was loaded on SDS-PAGE and after electrophoresis proteins were transferred to PVDF membranes by tank blotting (Biorad). Membranes were stained with anti-FLAG (Sigma polyclonal rabbit) to detect UL33ex1-FLAG expression. Loading on the gel was controlled by actin staining. Western blots were detected using anti rabbit-HRP conjugated secondary antibodies (BioRad) and chemiluminescent ECL substrate.

**Patient Samples and Immunohistochemistry.** Paraffin-embedded primary brain tumor specimens were available from 25 GBM patients who were admitted to the Karolinska University Hospital Sweden during 2009-2010. Tissue sections (6 μm thick) were stained for UL33 using sensitive immunohistochemistry staining protocols as previously been described [274]. Briefly, the sections were deparaffinized in xylene (Sigma Aldrich), rehydrate in alcohol series, postfixed with 4% neutral buffered formalin (Apoteketpharmaci, Stockholm, Sweden), treated with pepsin (Biogenex, San Ramon, CA), and then incubated in citrate buffer (Biogenex). The sections were treated with 3% H2O2 (Sigma-Aldrich) to inactivate endogenous peroxidase, avidin/avidin blocking kit (DakoCytomation, Glostrup, Denmark), was used in order to block endogenous biotin/avidin and FC receptor blocker (Innovex Biosciences) to block FC-R. Finally, the tissue sections were treated with background buster (Innovex Biosciences). Incubation with primary polyclonal rabbit antibodies against human CMV UL33 [145] was done overnight at 4°C. Polyclonal rabbit IgG antibodies (R&D Systems, Minneapolis, MN) was used as isotype control. After incubation with primary antibodies, the sections were incubated with biotinylated anti-rabbit (DakoCytomation) antibodies. Finally, the antibodies were visualized using streptavidin-conjugated horseradish peroxidase and diaminobenzidine (Innovex Biosciences). This study was approved by the ethics committees at Karolinska Institutet, Stockholm, Sweden (Dnr 2008/628-31/2).

**Acknowledgements**

We thank Stefan Dekker for technical assistance. Professor Wade Gibson is acknowledged for sharing the anti-UL33 antibody. This work was supported by The Netherlands Organization for Scientific Research (NWO) to E. Langemeijer, E. Slinger, D. Maussang and M.J. Smit, VIDI grant to M.J. Smit 700.54.425.