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Phenotypic Variation in Aicardi–Goutières Syndrome Explained by Cell-Specific IFN-Stimulated Gene Response and Cytokine Release

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Aicardi–Goutières syndrome (AGS; MIM 225750) is a genetically determined neurological disorder that typically becomes apparent in infancy (1). AGS encephalopathy is characterized by basal-ganglia calcification, white matter abnormalities, and a chronic cerebrospinal fluid (CSF) lymphocytosis. Patients with AGS suffer from microcephaly, spasticity, dys-}

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Abbreviations used in this article: AGS, Aicardi–Goutières syndrome; CSF, cerebrospinal fluid; EdU, 5-ethynyl-2′-deoxyuridine; hCMVEC, human cerebral microvascular endothelial cell; HEK, human embryonic kidney; hNSC, immortalized human neural stem cell; ISG, IFN-stimulated gene; LV, lentiviral vector; MOI, multiplicity of infection; NT, nontargeting; sRNA, short hairpin RNA; VEGF, vascular endothelial growth factor.

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Trex1-null mutant mice exhibit a reduced survival and develop inflammatory myocarditis, but a cerebral phenotype has never been observed (10, 15). Ablation of Rnaseh2b in mice leads to early embryonic lethality due to elevated DNA damage and p53-dependent growth arrest (16, 17). Adar1-null mutations also result in a widespread apoptosis in many tissues, as well as embryonic lethality (18). In contrast, Samhd1-null mutant mice do not develop any obvious pathology or autoimmunity (19, 20). All of these AGS animal models fail to replicate cerebral AGS-like symptoms and are therefore not suitable to study the intricate complexities of AGS neuropathology. Thus, human in vitro models are needed to study the cerebral alterations occurring in AGS (21). These models provide a unique tool to study AGS brain-related disease that otherwise would be limited to postmortem studies or imaging.

Early neuropathological human studies already suggested that AGS might represent a primary microangiopathy, because vascular mineral deposits and cortical microinfarctions were occasionally found in postmortem brains (22, 23). Recent clinical reports have described the presence of cerebrovascular disease, including ischemic and hemorrhagic strokes, in AGS patients carrying mutations for different genes (24–28). These clinical observations tie in with findings from our group describing that human astrocytes chronically exposed to IFN-α decrease the expression and release of proangiogenic and vascular trophic factors (21). Taken together, these observations suggest that an inflammatory disturbance of vascular homeostasis may be important in the pathogenesis of AGS.

The present study aims to gain deep insight into the specific role of each AGS gene in the biology of two important cell types of the gliovascular unit, which is part of the blood–brain barrier. To this end, short hairpin RNAs (shRNAs) against specific AGS genes were delivered to astrocytes and endothelial cells using lentiviral particles. This strategy resulted in the permanent knockdown of the targeted AGS genes, allowing us to study AGS pathogenesis in human cells, to our knowledge, for the first time in detail. We found that AGS protein knockdown leads to an increased but differential cytokine and IFN expression for both cell types tested. Interestingly, this effect was most dramatic after targeting TREX1 in both astrocytes and endothelial cells, which fits with the observation that patients carrying mutations in TREX1 suffer from the most severe phenotype (2) and with the observation that Trex1-null mutant mice have a strong IFN signature, which is weaker in patients carrying mutations in Samhd1 (19, 20). Our approach revealed novel links between the disease severity and the production of proinflammatory mediators.

Materials and Methods

Cell culture

Immortalized human neural stem cells (ihNSCs), derived from neural stem cells from the diencephalic and telencephalic brain regions of one human fetus, were cultured and propagated as previously described (29). Briefly, neural spheres were seeded in Euruomed-N medium (Eurobio Europe, Warrington, U.K.) in SuperMix for 1 h at room temperature. After three washes in TBST, bands were visualized with the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). Protein content was determined using the BCA protein assay kit (Pierce). RNA was DNase I treated and used as a template to generate cDNA following the manufacturer’s instructions (Quantitect reverse transcription kit; Qiagen, Hilden, Germany) with a blend of oligo(dT) and random hexamer primers. The reverse transcriptase reaction was incubated at 42˚C for 30 min. The resulting cDNA was diluted 1:10 and served as a template in real-time quantitative PCR assays (SYBR Green PCR Master Mix; Applied Biosystems, Warrington, U.K.). Sequences of primers used are given in Supplemental Table II. GAPDH and β-actin were used as reference genes to normalize the assessed transcript levels of the target genes.

Western blot

Protein was isolated from cells by homogenization in lysis buffer (100 mM NaCl, 10 mM Tris-HCl [pH 7.6], 1 mM EDTA [pH 8.0]) supplemented with protease inhibitors PMSF (100 mg/ml) and aprotinin (0.5 mg/ml) mixture (Roche). Protein content was determined using the BCA protein assay kit (Pierce). Aliquots of the protein lysates were separated by SDS PAGE and transferred to nitrocellulose membranes. The membranes were blocked in 5% skim milk or 5% nonfat milk (BioRad) for 1 h at room temperature. After blocking, the membranes were washed with TBST (10 mM Tris, pH 7.4, 0.15 M NaCl, 0.1% Tween 20) and probed with the following primary antibodies: rabbit anti-TREX1 (1:300, Abcam, Cambridge, U.K., ab67820), rabbit anti-ADAR1 (1:3000, Sigma-Aldrich, HPA003890), and mouse anti-β-actin (1:4000, Developmental Studies Hybridoma Bank, Iowa City, IA, JLA20). The next day, the blots were washed with TBST (100 mM Tris-HCl [pH 7.4], 150 mM NaCl, with 0.2% Tween 20) and incubated with secondary Ab anti-rabbit IRDye 800 or anti-mouse Cy3 (1:2000, Jackson Immunoresearch Europe, Suffolk, U.K.) in Superblocks for 1 h at room temperature. After three washes in TBST, bands were visualized with the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). Cell lysates from recombinant proteins were used as positive control: TREX1 (pcDNA4.TREX1orf, gift of...
Inhibition of TREX1 by shRNA induces apoptosis in human astrocytes

To study the significance of AGS genes in the astrocyte homeostasis, hiNSCs were differentiated into astrocytes (Fig. 1A) and subsequently transduced with LVs expressing shRNAs targeting the different AGS genes. Afterward, the protein expression of TREX1, SAMHD1, RNASEH2A, and ADAR1 was examined and found to be reduced as compared with NT shRNA-transduced cells (Fig. 1B). Previously published data showed that deletion or knockdown of one of the three subunits of the RNASEH2 protein complex is sufficient for the elimination of its activity (16). Although we could not detect SAMHD1 protein in our astrocyte lysates, we were able to detect SAMHD1 mRNA. These findings are in accordance with the gene atlas analysis showing a minimal expression of SAMHD1 in neural tissues (36). Moreover, we observed a major decrease in SAMHD1 mRNA upon knockdown (Supplemental Fig. 1).

An early observation suggested that silencing TREX1 in astrocytes resulted in a decreased cell survival. Indeed, the higher the multiplicity of infection (MOI) we used, the lower was the number of surviving cells (Fig. 1C). The effect was already evident with a low MOI, excluding a general toxic effect of the TREX1 shRNA. At higher MOIs, the number of TREX1 shRNA-transduced cells was decreased up to 70% compared with the NT shRNA control (p < 0.01, Fig. 1C, 1D). This effect on survival was not paralleled in the other control or AGS gene shRNA-transduced cell cultures. Only ADAR1 shRNA-transduced cells presented a slight, non-significant reduction. Because many floating cells were found in TREX1-transduced cultures, the cells were also stained against cleaved caspase-3. Indeed, we found a high number of positive cells, we determined the proportion of cells in each phase of the cell cycle during the short pulse labeling and therefore overcomes confounders such as cell death (37).

Silencing of TREX1 results in a reduced proliferation of endothelial cells

To assess the importance of AGS genes in human brain-derived microvascular endothelial cells, hCMEC/D3 cells were transduced with LVs expressing shRNAs targeting the different AGS genes (Fig. 2A). The expression of TREX1, SAMHD1, RNASEH2A, and ADAR1 was significantly reduced in the knockdown cells compared with NT shRNA-transduced cells at mRNA (Supplemental Fig. 2) and protein levels (Fig. 2B).

Next, we studied the effect of silencing AGS genes on the proliferation of hCMEC/D3 cells. Transduction with the different LVs had no visible effect on the cells, and cell death was not observed. However, we did notice a reduced proliferation of TREX1 shRNA-transduced cells. To further investigate this, we used Edu to pulse label cells for 2 h, allowing Edu to incorporate into all dividing cells. We then quantified the amount of Edu integration by flow cytometry (Fig. 2C). This method enumerates the proportion of label cells for 2 h, allowing EdU to incorporate into all dividing cells.

Results

Inhibition of TREX1 by shRNA induces apoptosis in human astrocytes

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Statistical analysis

All experiments were performed at least three independent times unless otherwise specified. Graphs are presented as bar charts with ± SEM. The IFN score was calculated from the median fold change value for a panel of seven IFN-stimulated genes (IFI17, IFI172, IFIT3, IFI39, OAS1, IFIT2, and RASAD2) as described previously (14). The variable distribution was assessed by the Kolmogorov–Smirnov test. When the test distribution was not normal,
quantitative FACS analysis revealed only a minor reduction in the proportion of TREX1 shRNA-transduced cells in M/G2 phase compared with control cells (Fig. 2F).

**AGS genes are not essential for in vitro vasculogenesis**

Endothelial cell migration and tube formation in Matrigel are widely used as in vitro assays for angiogenesis (38). To show the involvement of AGS gene silencing in capillary morphogenesis, endothelial knockdown cells were seeded on a Matrigel matrix (Fig. 3A). We analyzed the tube formation by measuring the length of the tubules (Fig. 3B) and the number of rings (Fig. 3C) formed by the cells on the matrix. We observed no differences in the knockdown cells compared with controls, demonstrating that silencing AGS genes does not affect the tubulogenic capacity of endothelial cells in vitro.

**FIGURE 1.** Effect of knockdown on ihNSC-derived astrocytes. (A) Immunostaining of cells against the astrocytic markers GFAP (in green) and S100B (in magenta). Nuclei are counterstained with Hoechst (in blue). Phase-contrast images are from cells transduced with AGS shRNAs. (B) Representative immunoblots from cell lysates confirm the knockdown of TREX1, SAMHD1, RNASEH2A, and ADAR1, respectively. Cell lysates from cells overexpressing the respective recombinant human protein were loaded as a positive control (+). Actin was detected as loading control. (C) First column of images corresponds to endogenous fluorescence of cells expressing GFP (in green) after transduction with the positive control SHC003-TurboGFP. Panels show immunostainings of transduced astrocytes against GFAP (in red) with increasing MOI (range, 1–50). (D) Graph shows quantification of number of cells per field after transduction with the different shRNAs (n = 3). Data represent means ± SEM. **p < 0.01. (E) Immunostaining of NT- and TREX1 shRNA-transduced cells against GFAP (in white) and cleaved caspase-3 (in green). Nuclei are counterstained with Hoechst (in purple). Caspase-3+ cells are indicated by arrowheads. Scale bars, 100 μm.
Interestingly, shRNA silencing of TREX1 in endothelial cells resulted in a reduced migration of those cells compared with control cells (Fig. 3D, 3E) after 24 h ($p < 0.05$) and 48 h ($p < 0.001$). TREX1 shRNA-transduced cells also showed a reduced migration capacity compared with other shRNA-treated cells after 48 h ($p < 0.001$).

Silencing AGS genes activates the expression of ISGs

Increasing evidence exists that IFN-α is not only a potent biomarker in AGS, but also a key player in AGS pathogenesis. We therefore studied the production of IFN-α and the ISGs in astrocytes and endothelial cells after silencing AGS genes.

IFN-α expression was induced in astrocytes after silencing of TREX1 ($p < 0.05$, Fig. 4A) but not after knocking down the expression of the other AGS genes. Likewise, the expression of MHC I (HLA-C), which is known to be induced by IFN-α, was increased only in TREX1 knockdown cells ($p < 0.05$, Fig. 4B). In contrast, endothelial cells showed no induction of either IFN-α or HLA-C upon silencing of any AGS gene (Fig. 4C, 4D).

When the expression of a panel of ISGs was studied, we observed increased $OAS1$ expression in astrocytes after silencing TREX1, SAMHD1, or ADAR1, but not after silencing RNASEH2A (Fig. 4E). Also, the overall IFN score was not increased in astrocytes compared with control shRNA-transduced cultures (Fig. 4F). The IFN score was calculated with the median fold changes of seven ISGs, as indicated in Materials and Methods, and represents the responsiveness to IFN. In contrast, we observed a significant increase in the mRNA expression of IFIT1 ($p < 0.05$), IFIT2 ($p < 0.05$), IFIT3 ($p < 0.05$), IRF9 ($p < 0.01$), OAS1 ($p < 0.05$), IFIT7 ($p < 0.05$), and RSAD2 ($p < 0.05$) in TREX1 knockdown endothelial cells (Fig. 4G). SAMHD1 knockdown endothelial cells also showed an increased expression of transcripts for IFIT2 ($p < 0.01$), IFIT3 ($p < 0.05$), and IRF9 ($p < 0.05$). No effects, however, were seen in the case of ADAR1 or RNASEH2A silencing on endothelial cells. The overall IFN score for TREX1-depleted cells was higher than that for NT shRNA control cells (Fig. 4H).

Silencing of TREX1 results in an increased production of proinflammatory and chemotactic cytokines

In addition to IFN-α, the chemokine CXCL10 has also been described to be elevated in CSF and plasma from AGS patients. CXCL10 expression is highly induced in astrocytes after silencing of TREX1 ($p < 0.01$), SAMHD1 ($p < 0.001$), and ADAR1 ($p < 0.05$; Fig. 5A). However, the induction of CXCL10 after silencing of RNASEH2A was much weaker compared with all other genotypes. Noticeably, only the ablation of TREX1 resulted in a clear induction of CXCL10 expression in endothelial cells ($p < 0.05$; Fig. 5B).

We also measured the cytokines and chemokines released by astrocytic and endothelial cell cultures after 24 and 7 d, respectively. In astrocytes, silencing of TREX1 resulted in a moderate release of the cytokine IL-6 ($p < 0.05$), as well as a substantial release of the chemokines CXCL10 ($p < 0.05$) and CCL5 ($p < 0.05$; Fig. 5C). These results are similar to those found in endothelial cells (Fig. 5D). Silencing of SAMHD1 only produced an increased release of CXCL10 ($p < 0.01$) and CCL5 ($p < 0.01$) in astrocyte cultures but not in endothelial cells. Silencing of ADAR1 or RNASEH2A did not induce any obvious increase in the production of cytokines in these cultures.
Silencing of TREX1 impairs the growth of primary human astrocytes in vitro and activates the expression of ISGs

To confirm the significance of AGS genes in the astrocyte homeostasis, the different AGS genes were knocked down in human primary astrocytes (Fig. 6A). The expression of TREX1, SAMHD1, RNASEH2A, and ADAR1 was examined and found to be reduced as compared with NT shRNA-transduced cells (Fig. 6B). As observed previously in the ihNSC-derived astrocytes, the final number of TREX1 shRNA-transduced human primary astrocytes cells was severely decreased compared with the NT shRNA control (p < 0.05, Fig. 6C).

Additionally, we measured the expression of IFN-α and other ISGs. IFN-α expression was induced in primary astrocytes only after silencing of TREX1 (p < 0.05, Fig. 6D). When we studied the expression of a panel of ISGs, we only observed increased IFIT2 expression after silencing SAMHD1 (p < 0.05, Fig. 6E).

Discussion

An important and fundamental aspect that remains to be clarified in AGS pathogenesis is the relationship between innate immune response, vasculopathy, and demyelination. It is unknown why a genetically heterogeneous disorder with mutations in at least six different, ubiquitously expressed genes leads to a syndrome with similar clinical manifestations and tropism for the CNS. Although the clinical picture of AGS patients may be alike in some features, these different mutations produce a diverse spectrum of phenotypes, in particular disease progression, with certain genotype/phenotype correlations. Patients carrying TREX1 mutations present a more severe clinical picture (presenting with abnormal neurology already in the neonatal period) and higher mortality rate compared with patients carrying an RNASEH2B mutation (2). In another study, researchers described a distinctive CSF lymphocytes gene expression signature in AGS patients bearing different gene mutations (39), and again in the present study, patients with mutations in TREX1 had the most severe and the earliest symptom onset. Furthermore, it has been shown that patients with SAMHD1 mutations have a later disease onset and a generally milder phenotype (24–26).

Our study clearly shows that silencing TREX1 resulted in a reduced viability of both ihNSC-derived astrocytes and primary
human astrocytes, as well as a reduced proliferation of endothelial cells. Silencing of any of the other AGS-causative genes did not parallel the strong TREX1-related effects. The most severe effect was observed after silencing TREX1 in astrocytes and, to our knowledge, this is the first time that TREX1 deficiency has been linked to cell death. Previous data on silencing TREX1 in murine

FIGURE 4. ISG signature activation on transduced cells. Bar graphs show IFN-α (A and C) and HLA-C (B and D) transcript levels in AGS gene shRNA-transduced astrocytes (n = 7, in black) and endothelial cells (n = 5, in gray). Normalized values are presented as fold over NT shRNA controls. (E) Quantitative PCR analysis of ISG mRNA expression in transduced astrocytes. Results are presented as fold over control (dashed line) and represent means ± SEM (n = 7). (F) IFN score was calculated from the median fold change from six ISGs. (G) Quantitative PCR analysis of ISG mRNA expression in transduced endothelial cells. Results are presented as fold over control (dashed line) and represent means ± SEM (n = 5). (H) TREX1 knockdown cells show an increased IFN score. *p < 0.05, **p < 0.01, ***p < 0.001.
and human fibroblasts reported an impaired G1/S cell cycle transition provoking a cell cycle arrest but not apoptosis (8). Increased apoptosis has never been reported in vivo in \textit{Trex1}-null mutant mice (9) or in their cells cultured ex vivo (10, 40). It is also intriguing why we observe this effect so dramatically in human astrocytes and not in endothelial cells. We do describe a reduced proliferation in human \textit{TREX1}-shRNA-transduced endothelial cells. This is in line with a previous study reporting that \textit{TREX1}-null mutant fibroblasts showed a chronic DNA damage checkpoint activation and a cell cycle arrest (8). Importantly, note that endothelial cells showed a reduced proliferation even though they are immortalized.

Other investigations have shown that also other AGS gene defects can affect proliferation. \textit{SAMHD1}-null mutant fibroblasts have been shown to exhibit a cell cycle arrest (41). Another investigation also revealed a delayed cell cycle progression after transient knockdown of \textit{SAMHD1} in HeLa cells using small interfering RNA (42). Alternatively, transient knockdown of \textit{ADAR1} in HeLa cells did not alter the cell growth (43). Hence, the effect of AGS gene deficiency seems to be both tissue and cell specific.

During the last few years, it has become clear that vasculopathy is an essential feature of AGS pathophysiology. From our results, we can conclude that AGS gene mutations have only a limited effect on the normal angiogenic, vasculogenic, and proliferative capacity of cerebral endothelial cells. This means that the vascular alterations observed previously by our group (21) and others (22) are most likely not intrinsically caused by the effect of the mutations in endothelial cells, but rather are caused by the environment. There are abundant data supporting this idea. First, AGS patients present substantial high levels of circulating IFN-\(\alpha\) (11). IFN-\(\alpha\) is a well-known potent antiangiogenic factor and is therefore used as coadjuvant therapy in different types of cancer (44). Second, the levels of IFN-\(\alpha\) remain sustainably high for years in both serum and CSF (12, 45). Third, a general downregulation of angiogenesis-related genes has been described for AGS patients carrying different genotypes (46). Fourth, we have previously shown that IFN-\(\alpha\) inhibits the production proangiogenic factors such as VEGF and IL-1 by astrocytes (21), the only VEGF-producing cells in the adult brain (47). Finally, we confirmed the downregulation of VEGF and IL-1 in AGS brain specimens (21). Taken together, these data suggest that the sustained alteration of the pool of pro- and antiangiogenic factors may lead to an abnormal vessel formation and proliferation profile in the brain of AGS patients.

Our data also show an induction of IFN-\(\alpha\) specifically in \textit{TREX1}-null mutant astrocytes and not in any other shRNA-treated cells. In fact, endothelial cells did not produce IFN-\(\alpha\) after transduction with any of the shRNA particles. In endothelial cells, the expression of many ISGs is induced independently of IFN-\(\alpha\). Many ISGs

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**FIGURE 5.** AGS gene knockdown induces the production and release of cytokines. Quantitative PCR analysis of \textit{CXCL10} mRNA expression in AGS gene shRNA-transduced astrocytes (A) \((n = 7,\text{ in black})\) and endothelial cells (\(n = 5,\text{ in gray}\)) (B). Normalized values are presented as fold over NT shRNA controls. (C) Secreted cytokines levels were measured in the supernatant from transduced astrocytes. Results are expressed in picograms per microgram total protein (mean \(\pm\) SEM, \(n = 9\)). (D) Cytokine and chemokine levels were measured in the supernatant from transduced endothelial cells. Results are expressed in picograms per microgram total protein (mean \(\pm\) SEM, \(n = 7\)). \(*p < 0.05, **p < 0.01, ***p < 0.001.**
IFIT1 can be easily activated by IRF proteins independently of IFN-α (48), and independent activation of ISGs has also been demonstrated most recently in Trex1-null mutant and Trex1 knockdown mouse embryonic fibroblasts (49). Our data clearly demonstrate that the knockdown of AGS genes differentially affects distinct cell types. Our findings support the unexplained findings in AGS patients, where the induction of ISGs such as IFIT1, IFIT2, IFIT3, IRF3, and RSAD2 was detected in TREX1-mutated fibroblasts and not in SAMHD1- and RNASEH2C-mutated cells (49). In contrast, the same measurement in whole blood cells from AGS patients showed many ISGs independent of the genotype (14). In this latter study, only a few patients carrying mutations in RNASEH2B gene appeared negative for the IFN response signature.

Even though IFN-α is considered to be a key biomarker for AGS, the chemokine CXCL10 has also been persistently demonstrated to have an elevated expression in AGS patients (12, 13). Consistent with this, our study shows that silencing AGS genes results in an increased expression and release of CXCL10 mainly in astrocytes and endothelial cells after silencing RNASEH2A. However, it remains unexplained why the ISG-activation signature is stronger in TREX1-null mutant cells in comparison with the other genotypes.

Even though IFN-α is considered to be a key biomarker for AGS, the chemokine CXCL10 has also been persistently demonstrated to have an elevated expression in AGS patients (12, 13). Consistent with this, our study shows that silencing AGS genes results in an increased expression and release of CXCL10 mainly in astrocytes. Furthermore, our work has also provided insight into the differential profile of cytokines in the different AGS genotypes. Also in the present study, TREX1-null mutant cells were producing the highest levels of cytokines. Collectively, the cytokine profiles seamlessly paralleled the ISG expression. Our data have shed some light on the functional implications of an ISG activation in the context of AGS. TREX1-null mutant cells present a more robust induction of ISGs that is translated into a higher expression and release of inflammatory cytokines and chemoattractants.

It has been suggested before that an IFN genetic signature may correlate with the phenotypic observations and clinical status in patients (14). We propose a model that links the chronic activation of ISGs with the cerebral autoimmune presentations observed in AGS, in which cytokines play a central role. First, patients carrying AGS gene mutations present an induction of ISGs and a sustained secretion of cytokines and chemokines. This continuous response stimulates the activation of the vasculature and facilitates the transmigration of inflammatory cells into the cerebral parenchyma. The persistent activation of the inflammatory cells might initiate demyelination. Additionally, the inflammatory disturbance of the brain vasculature may lead to prothrombotic phenomena and hypoperfusion that can contribute to demyelination. The disease severity may be reflected by higher ISG induction and cytokine secretion, with the worst prognosis in patients with a TREX1 mutation (2, 14). Future studies to confirm this hypothesis are needed.
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Disclosures

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