B-cell Expansion and Lymphomagenesis Induced by Chronic CD40 Signaling Is Strictly Dependent on CD19

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Abstract

CD40, a member of the TNF receptor family, is expressed on all mature B cells and on most B-cell lymphomas. Recently, we have shown that constitutive activation of CD40 signaling in B cells induced by a fusion protein consisting of the transmembrane part of the Epstein–Barr viral latent membrane protein 1 (LMP1) and the cytoplasmic part of CD40 (LMP1/CD40) drives B-cell lymphoma development in transgenic mice. Because LMP1/CD40-expressing B cells showed an upregulation of CD19, we investigated CD19's function in CD40-driven B-cell expansion and lymphomagenesis. Here, we demonstrate that ablation of CD19 in LMP1/CD40 transgenic mice resulted in a severe loss and reduced lifespan of mature B cells and completely abrogated development of B-cell lymphoma. CD19 is localized to lipid rafts and constitutively activated by the LMP1/CD40 fusion protein in B cells. We provide evidence that the improved survival and malignant transformation of LMP1/CD40-expressing B cells are dependent on activation of the MAPK Erk that is mediated through CD19 in a PI3K-dependent manner. Our data suggest that constitutively active CD40 is dependent on CD19 to transmit survival and proliferation signals. Moreover, we detected a similarly functioning prosurvival pathway involving phosphorylated CD19 and PI3K-dependent Erk phosphorylation in human diffuse large B-cell lymphoma cell lines. Our data provide evidence that CD19 plays an important role in transmitting survival and proliferation signals downstream of CD40 and therefore might be an interesting therapeutic target for the treatment of lymphoma undergoing chronic CD40 signaling.


Introduction

CD40, a member of the TNF receptor (TNF-R) family, is critically involved in germinal center formation and class switch recombination during T-cell dependent (TD)-immune responses (1). CD40 stimulation of B cells in vitro promotes B-cell activation, proliferation, and survival (2). CD40 is widely expressed on B-cell lymphomas, frequently along with the CD40 ligand (CD40L), resulting in constitutive engagement of CD40 (3, 4). In addition, mutations leading to constitutive activation of CD40 have been observed in multiple myelomas (5). We have shown recently that constitutively active CD40 signaling leads to B-cell activation and expansion and finally to B-cell lymphoma development in mice with high penetrance (6). These data suggest an important function of CD40 in B-cell lymphomagenesis.

The B-cell coreceptor CD19 is expressed from the early B-cell stages onwards. CD19-deficient mice display a severe reduction of marginal zone and B1 and a minor reduction of follicular B-cells (7–9). In the absence of CD19, germinal centers cannot be formed and antigen-specific IgG titers are strongly reduced underlying the essential role of CD19 in T-cell dependent-immune responses (7–9). Upon B-cell receptor (BCR) signaling, CD19 is known to directly interact with PI3K, Lyn, Grb2, Vav, and phospholipase C via phosphorylated tyrosine residues mediating signaling to Ca2+ release and PI3K (10–16). Pten deficiency was shown to fully restore marginal zone B and B1 cell development as well as germinal center formation in CD19 knockout mice, corroborating a prominent role for PI3K in CD19 signaling (17).

Because physiologically both CD19 and CD40 play a crucial role during B-cell activation and T-cell dependent-immune responses (1, 8, 18), it is particularly interesting whether CD19 is functionally involved in the pathogenesis of human lymphomas originating from aberrant chronic CD40 signaling (3). In the current study, we investigated the interplay of constitutive active CD40 signaling and CD19 in transmitting survival and proliferation signals. We show that deletion of CD19in LMP1/CD40 mice leads to a reduction of mature B-cell numbers and to the complete abrogation of B-cell lymphoma development. We found that CD40 requires CD19 to activate
PI3K and Erk signaling and that CD19 is phosphorylated in diffuse large B-cell lymphoma (DLBCL) cell lines, resulting in PI3K-dependent Erk activation. Our data suggest that CD19 is acting as a coreceptor not only for the BCR, but also for CD40 and mediates critical survival and proliferation signals.

Materials and Methods

Mice

Gt(Rosa26)Sor tm2(Lmp1/CD40)Uas (designated in the text as LMP1/CD40topfl) is described in ref. 6 and Cd19 tm1(cre)Cgn (designated in the text as CD19-Cre) mice in ref. 19. Mice were analyzed at 8 to 16 weeks of age unless stated otherwise. Mice were bred and maintained in specific pathogen-free conditions and experiments were performed in compliance with the German animal welfare law and have been approved by the institutional committee on animal experimentation. DLBCL cell lines used in this study are described in the figure legend of Supplementary Fig. S5B.

In vitro culture

Splenic B cells were purified by CD43 + depletion by “MACS” purification according to the manufacturer’s protocol (Miltenyi) as described (6). CD40 stimulation was performed with an agonistic CD40 antibody clone HM40–3 in a concentration of 1.25 to 2.5 μg/mL purchased from eBioscience. For Western blot analysis, MACS purified B cells or DLBCL- cell lines were left untreated or treated with inhibitors 1 hour before further procedures. Inhibitors: Ly294002, wortmannin, UO126, and sorafenib from Bayer HealthCare. The inhibitors were used in the following concentrations if not otherwise stated: Ly294002 20 μmol/L, wortmannin 0.1 μmol/L, UO126 10 μmol/L, sorafenib 10 μmol/L, and dasatinib 0.1 μmol/L.

Flow cytometry

Analyses were made with a FACSCalibur (BD Biosciences) as previously described (6). Results were analyzed using CELLQuest or FlowJo software. Antibodies used to stain cells for FACS analysis were all purchased from BD Biosciences (murine cells) or BioLegend (human cells).

Protein isolation and immunostaining

Western blotting with whole-cell extracts from purified B cells and BIC were performed as described recently (6). Antibodies for Western blot analyses: anti-p105/p50, anti-IkBa (sc-371), anti-CD40 (C-20) antibodies were purchased from Santa Cruz Biotechnology, anti-pCD19 (Y513), anti-pLyn, anti-pLyn (Y507), anti-Pten, anti-pY32/36, anti-Ba (sc-13522), anti-CD44, anti-CD40L, anti-CD4, anti-CD45, anti-CD19, anti-CD138, anti-pLyn (Y507), anti-Munc13-4, anti-pCD19 (Y513) antibodies were purchased from Santa Cruz Biotechnology, anti-pCD19 (Y513), anti-pLyn, anti-pLyn (Y507), anti-PTEN, anti-tubulin and anti-ε-Myc from Cell Signaling Technology, anti-GAPDH antibody from CALBIOCHEM.

Cryosections were processed, stained and analyzed as previously described (6, 20).

Lipid raft extraction

Purified B cells were incubated in Brij lysis buffer for 30 minutes on ice (1% Brij, 150 mmol/L NaCl, 20 mmol/L Tris/ HCl, pH 7.5, 2 mmol/L EDTA, freshly added protease, and phosphatase inhibitors) and lysed by douncing. Lipid rafts were isolated by ultracentrifugation (SW41 rotor in a Beckman Ultracentrifuge, 41,000 rpm, 4 °C, 16 hours) in a sucrose gradient (42.5%, 35%, 5%). Fractions 1–11 were saved and tested for GM-1 by probing with horseradish peroxidase (HRP)-conjugated cholera toxin subunit B (CALBIOCHEM). Proteins were then precipitated from the fractions by trichloroacetic acid, resuspended in 2× Laemmli buffer, denatured at 95°C for 5 minutes, and subjected to Western blot analysis.

In vivo BrdUrd assay

Mice were fed with 0.8 mg/mL bromodeoxyuridine (BrdUrd; Sigma) in the drinking water for 14 days. The water was exchanged every 2 to 3 days. Lymphocytes were isolated from the blood by Pancoll gradient centrifugation according to the manufacturer’s protocol (PAN). BrdUrd incorporation was analyzed by the APC BrdU Flow Kit (BD Biosciences).

Statistical analysis

Significance was calculated by the two-tailed student t test (\( \alpha < 0.05 \); \( \alpha < 0.01 \); \( \alpha < 0.001 \). Each experiment was performed at least three times if not otherwise stated.

Results

CD19 is required for premalignant LMP1/CD40-mediated B-cell expansion in vivo

Analysis of premalignant B cells from young LMP1/CD40topfl//Cd19-cre (LMP1/CD40) transgenic mice showed that CD19 expression levels are elevated in comparison with resting control B cells. (Fig. 1A). To analyze whether CD19 confers an advantage to LMP1/CD40-expressing B cells in vivo, LMP1/CD40 mice were crossed onto a CD19-deficient background by generating homozygous CD19-Cre mice (19). Splenomegaly, a hallmark of LMP1/CD40-expressing mice, was abrogated in age-matched LMP1/CD40//Cd19 cre mice (Fig. 1B). Calculation of total B-cell numbers in the spleen and inguinal lymph nodes (iLN) revealed significantly lower B-cell numbers in LMP1/CD40//Cd19 cre mice than LMP1/CD40//Cd19 +/+ mice (Fig. 1C), whereas developing B cells in the bone marrow were similarly represented in both genotypes (Supplementary Fig. S1). LMP1/CD40//Cd19 +/+ B cells still had a “blast-like” appearance and showed upregulation of B-cell activation markers such as CD95 and ICAM-1 (Fig. 1D). FACS analysis revealed that IgM + and IgD + B cells in the spleen and iLN of LMP1/CD40//Cd19 cre mice were strongly reduced in comparison with LMP1/CD40//Cd19 +/+ mice (Fig. 2A). The strong shift to the marginal zone B-cell phenotype (CD21 hi and CD23 low), which is characteristic for LMP1/CD40-expressing mice, was lost in the absence of CD19 (Fig. 2B). Only some residual marginal zone B cells could be detected by flow-cytometric analysis, but these cells did not localize to the marginal zone (Fig. 2C). CD19 deficiency in LMP1/CD40 mice strongly affects the expansion of both follicular B and marginal zone B cells (Fig. 2D). We concluded from these experiments that CD19 is strictly required for premalignant B-cell expansion in...
the periphery in young LMP1/CD40 mice, but not for CD40-induced expression of activation markers.

**CD19 is indispensable for LMP1/CD40-induced B-cell proliferation and survival**

To determine the survival of LMP1/CD40+/−/− B cells in vivo in comparison with LMP1/CD40+/−/+ B cells, we performed BrdUrd pulse-chase assays (21). At days 7 and 14 of the pulse period, CD19+/+, CD19+/−, and LMP1/CD40+/−/− mice showed similar percentages of BrdUrd+ B cells, whereas LMP1/CD40+/+ mice showed an increase of about 5- and 3-fold in blood and spleen, respectively (Fig. 3A). This is indicative of a higher turnover of LMP1/CD40+/−/− B cells compared with the other genotypes. The increased decline of BrdUrd-positive B cells in LMP1/CD40+/−/− mice during the chase from days 14 to 70 in blood and spleen (Fig. 3A) indicated a reduced lifespan of LMP1/CD40+/−/− B cells. In contrast, LMP1/CD40+/−/− B cells showed a survival advantage in comparison with control B cells as indicated by the higher number of BrdUrd-positive cells in the blood and spleen at day 70. Thus, CD19 expression is required for LMP1/CD40-mediated B-cell survival and proliferation. LMP1/CD40+/−/− and control B cells were isolated from the spleen and cultured in vitro for 5 days without stimulation to analyze their survival in vitro (Fig. 3B and Supplementary Fig. S2A). At days 1 and 2 of culture, cell numbers declined similarly after seeding of LMP1/CD40+/−/−, CD19+/+, and CD19+/−/− B cells and were significantly lower than those of LMP1/CD40+/+ B cells (Fig. 3B). Similarly the fraction of TO-PRO-3− LMP1/CD40+/−/− B cells declined rapidly in the first days of culture, whereas after 3 to 5 days the percentages of living cells increased again (Supplementary Fig. S2A), suggesting that a small percentage of B cells showed enhanced survival while the majority died within 3 days.

Proliferation was measured by carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling. At day 4 of splenic B-cell culture, the percentage of proliferating LMP1/CD40+/+ B cells averaged out at 3%, whereas LMP1/CD40+/−/− B cells contained around 10% proliferating cells (Fig. 3B). Similarly the fraction of TO-PRO-3− LMP1/CD40+/+ B cells declined rapidly in the first days of culture, whereas after 3 to 5 days the percentages of living cells increased again (Supplementary Fig. S2A), suggesting that a small percentage of B cells showed enhanced survival while the majority died within 3 days.

**Figure 1.** LMP1/CD40-mediated B-cell expansion in vivo is strictly dependent on CD19. A, the histogram shows an overlay of living cells of mice with indicated genotypes. The plot is gated on viable lymphocytes. The indicated color code at the bottom is valid for A–D. B, size of the spleen of mice with indicated genotypes. The graphs show total B-cell numbers in the spleen (Sp) and inguinal lymph nodes (iLN) of the indicated genotypes. Mean values and SDs are depicted that were calculated from at least 12 independent experiments. D, histograms show an overlay of cell size (forward scatter) and surface expression of ICAM-1 and CD95 from the indicated genotypes. The plots are gated on TO-PRO-3−, B220− cells.
Fig. 3E) and reduced rescue from apoptosis in response to CD40 stimulation (Supplementary Fig. S2B). These data point to an important role of CD19 in mediating survival and proliferation signals downstream of CD40. Next, we investigated whether deletion of CD19 in LMP1/CD40-expressing B cells abolishes B-cell lymphoma development. Twenty-two LMP1/CD40+/−/CD19+/−/− and 22 LMP1/CD40+/−/−/− mice were monitored for lymphoma development. Mice with suspected disease were sacrificed and lymphoid organs were analyzed for the presence of mono- or oligoclonal B-cell lymphomas (Supplementary Fig. S3). A high percentage of LMP1/CD40+/−/CD19+/−/− mice showed lymphoma development from the age of 11 months on, whereas none of the LMP1/CD40+/−/−/− mice did (Fig. 3F). This suggests that CD19 is required for LMP1/CD40-induced B-cell lymphoma development.

Activation of MAPK Erk by constitutive CD40 signaling is dependent on CD19 expression

Constitutive CD40 signaling in B cells in vivo leads to selective activation of the noncanonical NF-kB pathway as well as Jnk and Erk (6). LMP1/CD40+/−/CD19−/− B-cells showed increased p52 levels as seen in LMP1/CD40+/−/− but not in CD19+/−/− or CD19−/− B cells (Fig. 4A). Strikingly, both LMP1/CD40+/−/− and CD19−/− B cells showed a noticeable reduction of p100 levels, independent of their p52 levels. LMP1/CD40-expressing B cells further exhibited a reduction of pIκBα irrespective of their CD19 status. Erk phosphorylation...
was impaired in LMP1/CD40+/−CD19−/− compared with LMP1/CD40+/−/CD19−/− B cells, whereas the levels of pJnk were comparable (Fig. 4C). c-Myc levels were increased in LMP1/CD40+/−/CD19−/− but not in LMP1/CD40+/−/CD19−/− B cells (Fig. 4C), which might be either due to the impaired proliferation of LMP1/CD40 CD19-deficient B cells or to the decreased stability of c-Myc in the absence of CD19 as reported previously (22, 23).

**Figure 3.** CD19 mediates improved survival and enhanced proliferation downstream of CD40. A, pulse/chase BrdUrd experiment to determine the survival of B cells in vivo. Percentages of BrdUrd+/B220− B cells in the blood and the spleen were determined by FACS at the indicated time points. The graphs show the mean values of three independent experiments. B, splenic B cells were cultured for 5 days. Each day, cell numbers were determined. A and B, the symbols corresponding to the four different genotypes are valid for A and B. Asterisks indicate significant differences between the CD19-proficient and -deficient LMP1/CD40-expressing B cells. C, splenic B cells were labeled with CFSE before culture. At day 4, the proliferation profile of TO-PRO-3− cells was determined by FACS. The numbers indicate the percentage of proliferating B cells. D, splenic B cells were cultured for 7 days in the absence and presence of an agonistic CD40 antibody. E, each day, cell numbers were determined. Histograms showing the proliferation profile of TO-PRO-3− CFSE-labeled splenic CD19+/− and CD19−/− B cells after 4 or 7 days of culture in the presence of an agonistic CD40 antibody. F, loss of CD19 abrogates lymphoma development in LMP1/CD40 mice. The graph shows the lymphoma incidence in LMP1/CD40+/−CD19−/− and LMP1/CD40+/−CD19−/− mice aged up to 80 weeks.

**CD19 signaling is constitutively activated in LMP1/CD40-expressing B cells**

Because we found that CD19 is required for LMP1/CD40-mediated activation of Erk, we explored whether CD19 is constitutively activated in premalignant LMP1/CD40-expressing B cells. Upon antigenic triggering of the BCR, CD19 is recruited to lipid rafts (24) and phosphorylated on tyrosine residues Y513 and Y482 by Lyn. Upon binding to CD19, Lyn is
CD19 is phosphorylated in DLBCL cell lines

Klo and colleagues have shown that constitutive activation of the PI3K signaling pathway is a common feature of ABC DLBCL cell lines (27). Therefore, we investigated the status of CD19 phosphorylation in DLBCL cell lines of the ABC (U2932, HBL1, TMD8 RIVA, Oci-Ly3, and Oci-Ly10) and...
GCB subtypes (SU-DHL-6, SU-DHL-4, and BJAB). As controls, we used two Hodgkin lymphoma cell lines (KM-H2 and L428), which are CD19 negative. We detected phosphorylated CD19 in several DLBCL cell lines of the ABC and GCB subtype (Fig. 7A). Total CD19 levels varied between different cell lines being in tendency lower in ABC than in GCB-DLBCL cell lines (Fig. 7A and Supplementary Fig. S5B). We chose HBL1 and U2932, to analyze whether similar to LMP1/CD40-expressing B cells, Erk is activated through CD19 in a PI3K-dependent manner in DLBCL cell lines. The PI3K inhibitor LY294002 impaired Erk and Akt but not Jnk phosphorylation in both cell lines (Fig. 7B). The Raf inhibitor, sorafenib, was not able to reduce Erk phosphorylation, similar as in LMP1/CD40-expressing B cells (Supplementary...
Fig. S5A). CD19 phosphorylation as well as Erk and Lyn phosphorylation was abrogated in the presence of dasatinib in all cell lines tested, whereas sorafenib increased Erk activation (Fig. 7C). In contrast PI3K and Mek inhibition resulted in decreased Erk but not CD19 and Lyn phosphorylation, indicating that PI3K and Lyn are acting upstream of Mek. These data suggest that like in LMP1/CD40+/− B cells, active Lyn phosphorylates CD19, leading to Erk activation in a PI3K-dependent manner. After having established that dasatinib almost completely removes CD19 phosphorylation, we used this drug to determine the effect of CD19 phosphorylation on the survival of DLBCL cell lines. Except of two cell lines (SU-DHL-4 and Oci-Ly3), all cell lines were sensitive to the treatment with dasatinib and displayed a reduced survival in comparison to untreated control DLBCL cell lines (Fig. 7D). Of note, in the group of dasatinib-sensitive cell lines, those cell lines displaying lower levels of constitutive Erk phosphorylation were more sensitive for
dasatinib treatment. These data suggest that CD19/PI3K-dependent Erk activation is a prosurvival signaling pathway in DLBCL cell lines.

Discussion

CD19 has been shown to directly interact with, and to activate PI3K (11, 13, 14), the critical survival signal downstream of the BCR (28). Because CD19 was highly expressed on the surface of LMP1/CD40-expressing B cells, we investigated whether their improved survival depends on CD19. We found that ablation of CD19 has a severe effect on the survival and proliferation as well as lymphomagenesis of LMP1/CD40-expressing B cells. The reduced proliferation and increased apoptosis of CD19 deficient in comparison with control B cells in the presence of CD40 stimulation underline our assumption...
that CD19 transmits proliferation and survival signals downstream of CD40.

The failure of LMP1/CD40 to activate Erk in the absence of CD19 might be the reason for this phenotype, since we showed recently that Erk is crucial for the survival advantage of LMP1/CD40-expressing B cells (6). We show that Y513-phosphorylated CD19 is recruited together with LMP1/CD40 and pLyn into lipid rafts, suggesting its constitutive activation (24). Our data suggest that CD40-induced CD19 phosphorylation is mediated by the kinase Lyn because (i) Lyn is hyperphosphorylated in LMP1/CD40-expressing B cells; (ii) CD40 stimulation of B cells results in the activation of Lyn and CD19; and (iii) CD19 phosphorylation is inhibited by the chemical Lyn inhibitor dasatinib. We observed a strong downregulation of Pten in CD19-deficient B cells, which might reflect a rescue mechanism of CD19-deficient B cells, to maintain some basal PI3K activity. Our data suggest that CD19 is acting not only as a coreceptor for the BCR, but also for CD40. We postulate that phosphorylated CD19 activates PI3K and subsequently Akt, Mek, and Erk. We assume that BCR signaling is not involved in this activation process, because genetic truncation of the signaling tail of IgB does not reduce the constitutive Erk activation in LMP1/CD40-expressing B cells (own unpublished results). Akt might not directly activate Mek but rather uses additional signaling mediators for the activation of Mek and Erk. Because sorafenib treatment rather resulted in increased Erk activation, it is unlikely that Raf kinases are involved in activation of this signaling pathway. It might be that PI3K/Akt is acting through the IKK complex to activate Mek and Erk because links between PI3K and NF-kB as well as NF-xB and MAPK-signaling have already been described (27, 29).

CD19 is phosphorylated in several DLBCL cell lines of the ABC as well as GCB subtype. Like in LMP1/CD40-expressing B cells, the phosphorylation of Erk is dependent on Lyn, PI3K, and Mek activity in DLBCL cell lines. Abrogation of Lyn and downstream CD19 phosphorylation by dasatinib resulted in impaired survival of most DLBCL cell lines, suggesting that Lyn phosphorylation mediates through CD19 an important survival signal also in DLBCL cell lines. Only two cell lines, Oci-Ly3 and SU-DHL-4, were resistant to dasatinib treatment. One of these cell lines, Oci-Ly3 carries a constitutive CARD11 mutation, the other cell line, SU-DHL-4, an IgH-hcI2 translocation leading to the overexpression of Bcl2 (30). These mutations might circumvent the dependency on CD19 phosphorylation. We could not provide the final proof that similar like in LMP1/CD40-expressing cells, CD19 phosphorylation is mediated through CD40 signaling in DLBCL cell lines. Both chronic BCR and CD40 signaling might lead to CD19 phosphorylation in DLBCL cell lines. Chronic BCR signaling has been described in the ABC DLBCL cell lines HBL1, TM98, and Oci-Ly10 (30). Evidence for chronic CD40 signaling in some aggressive B-cell lineage non-Hodgkin's lymphomas (NHL-B) has been provided by Pham and colleagues (3). They described a "CD40 signaling-some," consisting of CD40, CD40L, TRAF2, and TRAF6 as well as components of the NF-xB signaling pathway that are localized in lipid rafts of NHL-B. Therefore, we analyzed CD40 and CD40-L expression in the DLBCL cell lines. All DLBCL cell lines expressed CD40 (Supplementary Fig S6A), but expression of CD40-L was either extremely low or undetectable (data not shown). Thus, it is unlikely that CD19 phosphorylation is mediated by CD40-CD40L auto-stimulation in DLBCL cell lines, but CD40 auto-aggregation might be responsible for CD19 phosphorylation in DLBCL cell lines. Probably, CD19 phosphorylation in DLBCL cell lines does not completely reflect CD19 phosphorylation in situ. Most DLBCL cell lines express high levels of CD40 (Supplementary Fig, S6A). It is therefore tempting to speculate that in vivo lymphoma cells constantly receive CD40 signals by lymphoma-infiltrating CD40-L-expressing cells such as activated T cells or myeloid cells, which might increase CD19 phosphorylation. Increased CD19 phosphorylation after CD40 stimulation of BJAB cells is underlying this hypothesis (Supplementary Fig S6B). Collectively, our data suggest an important role of CD19 in enhancing and sustaining chronic B-cell activation, survival, and proliferation. Because aberrant chronic activation of both BCR and CD40 signaling can contribute to B-cell lymphomagenesis (3, 6, 30, 31), CD19 may emerge as a critical player in this process. The important role of CD19 in lymphoma progression is underlined by the observations that in the absence of CD19, lymphoma development is completely abrogated in LMP1/CD40 mice and delayed in c-myc<sup>+</sup> mice (23). Our data provide evidence that molecules interfering with CD19 signaling might be valuable new tools in the treatment of B-cell lymphomas undergoing chronic BCR or CD40 signaling.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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