Role of STAT3 in Type I Interferon Responses

NEGATIVE REGULATION OF STAT1-DEPENDENT INFLAMMATORY GENE ACTIVATION*

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Type I interferons (IFNα/β) induce antiviral responses and have immunomodulatory effects that can either promote or suppress immunity and inflammation. In myeloid cells IFNα/β activates signal transducers and activators of transcription STAT1, STAT2, and STAT3. STAT1 and STAT2 mediate the antiviral and inflammatory effects of IFNα/β, but the function of IFNα/β-activated STAT3 is not known. We investigated the role of STAT3 in type I IFN signaling in myeloid cells by modulating STAT3 expression and the intensity of STAT3 activation using overexpression and RNA interference and determining the effects on downstream signaling and gene expression. IFNα-activated STAT3 inhibited STAT1-dependent gene activation, thereby down-regulating IFNα-mediated induction of inflammatory mediators such as the chemokines CXCL9 (Mig) and CXCL10 (IP-10). At the same time, IFNα-activated STAT3 supported ISGF-3-dependent induction of antiviral genes. STAT3 did not suppress STAT1 tyrosine phosphorylation or nuclear translocation but instead sequestered STAT1 and suppressed the formation of DNA-binding STAT1 homodimers. These results identify a regulatory function for STAT3 in attenuating the inflammatory properties of type I IFNs and provide a mechanism of suppression of STAT1 function that differs from previously described suppression of tyrosine phosphorylation. The results suggest that changes in the relative expression and activation of STAT1 and STAT3 that occur during immune responses determine the nature of cellular responses to type I IFNs.

The Janus kinase (JAK)2-signal transducer and activator of transcription (STAT) signaling pathway is the major signaling pathway activated by the type 1 (IFNα and IFNβ) and type II (IFNγ) interferons, key immune regulatory cytokines (1, 2). IFNγ, a potent inflammatory cytokine and macrophage activator, predominantly activates STAT1 that mediates the inflammatory, pro-apoptotic, and anti-proliferative effects of this cytokine (3, 4). IFNγ only weakly activates STAT3, which opposes the biological functions of STAT1 and mediates anti-inflammatory, anti-apoptotic, and proliferative effects (5–7). In contrast to IFNγ, the pleiotropic cytokine IL-6 activates both STAT1 and STAT3, and the anti-inflammatory cytokine IL-10 activates predominantly STAT3 (8, 9). STAT1 and STAT3 oppose each others activation by the IFNγ and IL-6 receptors, and this mutual antagonism results in an integrated signal that can be fine tuned depending on cellular context (5, 8, 10). Mechanisms of negative cross-regulation by STAT1 and STAT3 include competition for common receptor docking sites and STAT3-dependent activation of SOCS3 expression (5, 7, 10–16). SOCS3, in turn, regulates IL-6 signaling by binding to STAT3 and suppressing downstream STAT3 activation (7, 11–16).

Type I IFNs are pleiotropic cytokines that have potent antiviral activity and promote the transition from innate to acquired immunity, but can also suppress inflammatory responses and diseases such as multiple sclerosis and inflammatory bowel disease (2, 17–20). The receptor for type I interferons consists of two subunits, IFNAR-1 and IFNAR-2, that are associated with the JAK tyrosine kinases Tyk2 and Jak1, respectively, which are in turn responsible for downstream activation of multiple STATs. IFNα can activate all of the known STATs, but in myeloid cells activates predominantly STAT1, STAT2, and STAT3 (17, 20). Tyrosine-phosphorylated STAT1 and STAT2, together with IFN regulatory factor 9 (IRF-9), assemble into the heterotrimeric IFN-stimulated gene factor 3 (ISGF-3) complex that binds to the IFN-stimulated response element (ISRE) and initiates transcription that accounts for many of the antiviral and growth inhibitory properties of type I IFNs (17). In addition, IFNα stimulation leads to the formation of STAT1 homodimers that bind to γ-activated sequence (GAS) promoter elements and activate canonical IFNγ-induced STAT1-dependent genes (7, 21, 22). Activation of these genes can explain some of the immunomodulatory effects of type I IFNs. We have recently reported that increased activation of STAT1 by IFNα in IFNγ-primed macrophages resulted in increased activation of STAT1-dependent inflammatory genes, such as the chemokines CXCL9 (Mig) and CXCL10 (inducible protein–10) (22). One important determinant of increased STAT1 activation by IFNα in primed macrophages was the increased level of STAT1 expression relative to expression of STAT2 and STAT3.

IFNα strongly activates STAT3, but the functional consequences of this activation are not well understood. Roles for IFNγ-activated STAT3 in promoting T cell proliferation and in enhancing antiviral effects of IFNα/β in a B cell line have been proposed based on use of, respectively, a STAT3 inhibitor and complementation of B cell lines with STAT3 (23, 24). However, a genetic approach using cells deficient in STAT3 did not detect a role for STAT3 in IFNγ regulation of T cell proliferation (21), and the molecular basis for enhancement of antiviral responses by STAT3 has not been clarified. Gene expression profiling of IFNα responses has not demonstrated substantial activation of canonical STAT3 target genes or anti-inflammatory genes such as those activated by IL-10 (25–28), further questioning the role of STAT3 in mediating the biological properties of type I IFNs.

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† The abbreviations used are: JAK, Janus kinase; IFN, interferon; Mig, monokine induced by γ-IFN, interferon regulatory factor; CXCL, CXCL ligand; ISGF-3, IFN-stimulated gene factor 3; ISRE, IFN-stimulated response element; GAS, γ-activated sequence; STAT, signal transducer and activator of transcription; Mx2, myxovirus resistance 2; OAS, oligoadenylate synthetase; SOCS, suppressor of cytokine signaling; EMSA, electrophoretic mobility shift assay; IL, interleukin; RNAi, RNA interference; shRNA, short hairpin RNA; EMA, electrophoretic mobility shift assay; eGFP, enhanced green fluorescent protein.
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We hypothesized that one function of IFN-α-activated STAT3 is to regulate STAT1 activation or function, and that STAT3 mediates anti-inflammatory properties of type I IFNs by suppressing STAT1. We tested this notion by modulating the intensity of IFN-α activation of STAT3 and determining the effects on downstream signaling and gene expression. Our results demonstrate a role for IFN-α-activated STAT3 in suppressing IFN-α induction of STAT1-dependent inflammatory genes, such as CXCL9 and CXCL10, further testifying to the counterbalancing action of STAT1 and STAT3. In contrast to previously described STAT3-mediated mechanisms for suppressing STAT1 activation downstream of other receptors, STAT3 did not suppress STAT1 tyrosine phosphorylation but instead sequestered STAT1 and suppressed the formation of DNA-binding STAT1 homodimers. These results extend our understanding of STAT1-STAT3 cross-regulation in mediating cellular responses to cytokines and identify a function for STAT3 in type I IFN signaling.

EXPERIMENTAL PROCEDURES

Biological Reagents and Cell Culture—Recombinant human IFNα and IFNγ were purchased from Pepro Tech, Inc. (Rocky Hill, NJ). THP-1 human monocytic cells were obtained from ATCC and cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT).

Lentiviral Gene Transduction and RNA Interference (RNAi)—A lentivirus-based vector expressing the human STAT1 or STAT3 cDNA driven by a human phosphoglycerol kinase promoter was used to generate recombinant lentiviral particles as described (29). A construct that contained a transcription cassette encoding enhanced green fluorescent protein (eGFP) driven by the human phosphoglycerol kinase promoter was used to generate control viral particles for STAT1 and STAT3 expression experiments. For STAT1 and STAT3 RNA interference, oligonucleotides encoding several different short hairpin RNAs (shRNAs) that target human STAT1 or STAT3 were cloned into the lentivirus-based RNAi vector pLl3.7 that also contains a transcription cassette encoding eGFP driven by a cytomegalovirus promoter (29). Constructs that were effective in suppressing STAT1 or STAT3 expression were identified using transient cotransfection of HEK 293T cells with expression plasmids encoding the different STATs. The constructs that were most effective in HEK 293T cells (containing the shRNA sequences 5′-GCGTAATCTTCAGGATAAT-3′ or 5′-ACCTGGAGAGGAGGACAG-3′; for human STAT1 and 5′-AGTCAGGTTGCTGGTCAAA-3′ or 5′-GCATCGCTAGATCGGCTA-3′; for human STAT3) were used to generate recombinant lentiviral particles. Lentiviral particles encoding interfering shRNA against red fluorescence protein DSRed2 (5′-GTGG-GAGCCGGGTGATGAGC-3′) were used as a control for STAT1/STAT3 RNAi experiments. THP-1 cells were incubated overnight with recombinant lentiviral particles at a ratio of 1:50 in the presence of 4 μg/ml Polybrene. The efficiency of transduction was evaluated using flow cytometry and fluorescence microscopy to monitor eGFP expression and was typically >90%.

Real Time Quantitative Reverse Transcriptase-PCR—For real time PCR, total RNA was extracted using an RNeasy Mini kit (Qiagen, Valencia, CA) and 1 μg of total RNA was reverse transcribed using a First Strand cDNA Synthesis kit (Fermentas, Hanover, MD). Real time quantitative PCR was performed using iQTM SYBR Green Supermix and iCycler iQ™ thermal cycler (Bio-Rad) following the manufacturer’s protocols. Oligonucleotide primers used were as previously described (22, 30). Triplicate reactions were run for each sample and mRNA levels were normalized relative to β-actin. The generation of only the correct size amplification products was confirmed using agarose gel electrophoresis.

RESULTS

STAT3 Suppresses IFNα-induced STAT1-dependent Gene Activation—Negative cross-regulation by STAT1 and STAT3 was identified using cells deficient in STAT1s (5, 10, 33, 34). Because STAT1 and STAT3 are ubiquitously expressed, these experiments are subject to the caveat that they do not accurately mimic cell physiology. However, we and others have shown that STAT1 and STAT3 expression varies substantially in different cell types and in response to physiological stimuli, and that their relative expression impacts on biological responses in physiological settings (5, 8, 10, 30, 35, 36). Therefore, instead of using cells completely deficient in STAT1 or STAT3, we mimicked physiological regulation of STAT expression by modulating relative expression of STAT1 and STAT3 using a combination of forced expression and RNAi-mediated knockdown in THP-1 monocytic cells. This approach has the advantage of modulating STAT levels in THP-1 cells in the absence of any other effects that would be induced by cytokines that modulate STAT expression.

THP-1 cell lines were generated by transduction with lentiviral constructs encoding STAT1 or STAT3 or shRNAs that target STAT1 or STAT3 mRNA for degradation via RNA interference. Transduction efficiency was typically >90% as assessed by co-expressed eGFP; this approach allows rapid generation and analysis of cell lines without the potential artifacts associated with selection of transfected clones. At least three cell lines generated by independent transduction of each construct were analyzed and two different shRNAs that effectively suppress STAT1 and STAT3 expression were used. In experiments where transduction efficiency with shRNAs was less than 90%, transduced cells were further enriched using flow cytometric sorting based upon co-expressed eGFP. THP-1 cells transduced to express eGFP were used as a control for cell lines transduced to express STATs, whereas THP-1 cells transduced to express a shRNA that effectively knocks down DSRed2 expression were used as a control for cell lines transduced to express shRNAs. Fig. 1 shows representative results showing that this approach was successful in modulating STAT1 and STAT3 mRNA (Fig. 1A) and protein (Fig. 1B).
1B) levels over a broad range. Since it has been previously reported that certain small double-stranded RNAs can induce expression of antiviral genes in mammalian cells (37–39), we verified that the shRNAs used in this study did not significantly induce expression of the antiviral oligoadenylate synthetase (OAS) and myxovirus resistance protein-2 (Mx2) genes (data not shown). Activation of STAT tyrosine phosphorylation by IFNα was generally proportional to STAT expression, and is further addressed experimentally below.

Our eventual goal was to determine the effects of STAT3 on STAT1-dependent inflammatory IFNα responses, such as activation of the chemokine genes CXCL9 and CXCL10. We first needed to define the regulation of these genes by STAT1 in our system. Therefore, THP-1 cells expressing low or high STAT1 were stimulated with IFNα, which did not detectably activate STAT3 in THP-1 cells (data not shown), and thus allowed us to examine the effects of modulating STAT1 expression without potential confounding effects of activating STAT3. As expected (30), IFNα induced expression of STAT1 mRNA in a time-dependent manner in control THP-1 cells (Fig. 2A). In THP-1 cells expressing shRNA that targets STAT1 (hereafter termed STAT1lo cells), basal levels of STAT1 mRNA were lower than in control THP-1 cells, and the induction of STAT1 expression by IFNα was reduced (Fig. 2A). The activation of the IRF-1, CXCL9, and CXCL10 genes was reduced in STAT1lo THP-1 cells, and this reduction was most apparent at later time points (Fig. 2, A–D). These results are confirmatory of previous reports and demonstrate that these genes are STAT1-dependent in our system. In addition, the more effective suppression of gene expression observed at later time points suggests that induction of STAT1 expression contributes to the sustained and time-dependent increase in IFNα-induced gene expression that was observed (Fig. 2).

Next, the effects of decreased STAT1 expression on gene activation by IFNα were investigated. Induction of STAT1, IRF-1, CXCL9, and...
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CXCL10 mRNA by IFNα was reduced in STAT1lo relative to control THP-1 cells (Fig. 3). These results show that induction of these genes by IFNα is STAT1-dependent. Because IFNα strongly activates STAT3 in THP-1 cells (see below) and STAT3 can suppress STAT1 activation (5, 10–14, 17), we wished to test the role of STAT3 in down-regulation of IFNα-induced STAT1-dependent gene expression. The first approach was to use THP-1 cells that had been transduced with lentiviral constructs that express STAT3, hereafter termed STAT3hi THP-1 cells. Increased expression of STAT3 reduced IFNα induction of IRF-1, CXCL9, and CXCL10 mRNA (Fig. 4, A–C). Reduced induction of CXCL9 and CXCL10 mRNA in STAT3lo THP-1 cells was apparent over a broad range of IFNα concentrations (Fig. 4D and data not shown). STAT3 could reduce STAT1-dependent gene activation either directly or through the induction of inhibitory molecules such as SOCS3 (5, 10–13, 17, 40). The latter possibility was addressed by using cycloheximide to inhibit de novo protein synthesis and thereby block STAT3-dependent induction of putative inhibitory proteins. STAT3 reduced IFNα-induced STAT1-dependent gene expression in the presence of cycloheximide (Fig. 4, A–C), thereby suggesting a direct inhibitory effect of STAT3 on STAT1 function. We also assessed the IFNα induction of the OAS and Mx2 genes in STAT3hi THP-1 cells. In contrast to the IRF-1, CXCL9, and CXCL10 genes whose activation is mediated by a GAS element, IFNα induction of the OAS and Mx2 genes whose activation is mediated by an ISRE was not suppressed by STAT3, and there was a trend toward higher induction of OAS and Mx2 expression in STAT3hi THP-1 cells at later time points after IFNα stimulation (Fig. 4E). As expected, induction of the STAT3-dependent SOCS3 gene was enhanced in THP-1 cells that expressed high STAT3 levels (Fig. 4F). These results show specificity of STAT3-mediated inhibition and suggest that STAT3 is more effective in suppressing GAS-dependent transcription (dependent on STAT1 homodimers) than ISRE-dependent transcription (dependent on the STAT1- and STAT2-containing ISGF3).

To complement the overexpression approach, we examined IFNα induction of gene expression in THP-1 cells in which STAT3 expression had been knocked down using RNAi (hereafter termed STAT3lo THP-1 cells). We tested the notion that decreased STAT3 expression would lead to augmented IFNα induction of STAT1-dependent genes. As shown above in Fig. 1, these STAT3lo cells contain normal STAT1 levels but express only 10–20% as much STAT3 as control THP-1 cells (DSRed2-RNAi). Upon stimulation by IFNα, the STAT3lo cells displayed enhanced activation of all three STAT1-dependent genes evaluated here, IRF-1, CXCL9, and CXCL10, an effect that was also independent of new protein synthesis because it was cycloheximide resistant (Fig. 5, A–C). Enhanced induction of CXCL9 and CXCL10 mRNA in STAT3lo THP-1 cells was apparent over a broad range of IFNα concentrations (Fig. 4D and data not shown). In contrast, the induction of OAS and Mx2 by IFNα was attenuated in STAT3lo THP-1 cells (Fig. 5D), especially at the later time point (3 h). These results demonstrate that basal levels of STAT3 in THP-1 cells are sufficient to reduce STAT1-dependent GAS-driven gene expression. The results also further support a role for STAT3 in augmenting induction of ISRE-dependent genes that may contribute to the antiviral function of STAT3 previously documented in Daudi B cells by Yang et al. (24).

STAT3 Does Not Suppress Activation of STAT1 by IFNα—Our results show that STAT3 reduces IFNα induction of STAT1-dependent genes, which can occur either by inhibiting the activation or function of STAT1. Previous reports have shown that STAT3 can suppress activation of STAT1 by inhibiting tyrosine phosphorylation, which occurs in a cellular context- and signal-dependent manner, as STAT3 can inhibit STAT1 activation by IL-6 family cytokines (10, 11, 13, 14, 41), but does not suppress STAT1 activation by IFNγ in endothelial cells (42). Therefore, we investigated the role of STAT3 in modulating IFNα-induced STAT1 tyrosine phosphorylation. As expected, IFNα-induced STAT3 tyrosine phosphorylation correlated with STAT3 expression and was sub-

FIGURE 3. STAT1 dependence of IFNα-induced gene expression in THP-1 cells. THP-1 cells transduced with control DSRed2-shRNA- or STAT1-shRNA-encoding lentiviral particles were stimulated with 10,000 units/ml (25 ng/ml) of IFNα for 1, 3, or 24 h and mRNA levels were measured using real time PCR and normalized relative to β-actin. Results showing expression relative to unstimulated control cells (first bar) are expressed as mean ± S.D. of triplicate determinants.
Type I IFNs are pleiotropic immunomodulatory cytokines that have the potential to both promote and suppress inflammation. Inflammation is a complex process that involves the activation of various signaling pathways, including the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. This pathway is crucial for the regulation of immune responses and the induction of anti-inflammatory genes.

**Figures and Table: STAT3 Attenuates STAT1-mediated Gene Expression**

**A** shows the relative expression of IRF-1 in THP-1 cells treated with IFNα. **B** demonstrates the relative expression of CXCL9 in THP-1 cells treated with IFNα. **C** illustrates the relative expression of CXCL10 in THP-1 cells treated with IFNα. **D** presents the relative expression of CXCL9 in THP-1 cells treated with graded concentrations of IFNα. **E** shows the fold induction of OAS and Mx2 in THP-1 cells treated with OAS and Mx2. **F** displays the relative expression of SOCS3 in THP-1 cells treated with STAT3.

**Discussion:**

Type I IFNs play a critical role in regulating immune responses and inflammation. The development of strategies to target the JAK/STAT pathway, such as the use of STAT3 inhibitors, could be beneficial in the treatment of inflammatory diseases.
tory properties of type I IFNs can be attributed in part to the activation of STAT1 and many of the same STAT1 target genes as the potent cell activator IFN-γ. The major finding of this study is the identification of a role for IFNα-activated STAT3 in attenuating the function of STAT1 and thereby restraining the inflammatory properties of IFNα, as assessed by chemokine expression. The results support the emerging notion that the balance between activation of STAT1 and STAT3 by type I IFNs, and by other cytokines such as IFNγ, IL-6, and IL-10, determines the relative inflammatory potency of these cytokines (5, 10–13, 17, 35, 43, 44).

In myeloid cells, type I IFNs activate predominantly STAT1, STAT2, and STAT3 by tyrosine phosphorylation, with subsequent formation of the ISGF-3 complex (comprised of STAT1, STAT2, and IRF-9) and of STAT1/STAT3 homo- and heterodimers. The importance of ISGF-3 in antiviral responses is well established (17, 20), and we have recently implicated STAT1 in IFNα induction of inflammatory genes, including CXCL9 and CXCL10, in macrophages (22). The role of STAT3 in type I IFN signaling is not well understood as STAT3-dependent IFNα target genes and their functions have not been defined, and a role for IFNα/β-induced STAT3 in promoting cell survival and proliferation defined by inhibitor studies (23) was not detected by a genetic approach using STAT3-deficient cells (21). Our results suggest a regulatory function for STAT3, namely attenuation of STAT1-mediated inflammatory functions. At the same time, IFNα-activated STAT3 supported ISGF-3-dependent induction of antiviral genes. Thus, STAT3 appears to regulate type I IFN responses such that a potent antiviral response can occur in the absence of excessive inflammation that will cause collateral tissue damage. This stands in contrast to IFNγ, which activates STAT1 strongly relative to STAT3 and potently activates inflammation but induces a weaker anti-viral response.

The regulation of relative expression of type I and type II IFNs during the course of an immune response that is reflected by varying relative activation of STAT1 and STAT3 will determine the amount of inflammation associated with antiviral responses.

Cellular responses to type I IFNs are not fixed, but vary qualitatively depending on the activation state of the cell (Refs. 22 and 45; this study).
One important determinant of the nature of IFNα responses is the level of expression of STAT1. For example, priming of macrophages with low subactivating concentrations of IFNγ that increase STAT1 expression results in enhanced STAT1 activation by IFNα, with concomitant increased activation of inflammatory STAT1 target genes (22). Increased STAT1 expression during viral infection attenuates IFNα-mediated activation of STAT4 and IFNγ production and regulates CD8 T cell proliferation (45, 46). This previous work on modulation of IFNα responses focused on the role of increased STAT1 expression, which is itself induced by either IFNγ or IFNα/β, and has been detected not only in viral infections, but also in chronic autoimmune diseases such as rheumatoid arthritis, lupus, and dermatomyositis (27, 47–53). Our results showing a regulatory role for STAT3 in IFNα responses is the absence of new protein synthesis and thus inhibited STAT1 function directly without the need for synthesis of inhibitory molecules. We considered three potential mechanisms by which STAT3 could directly block the activation/function of STAT1. 1) Competition for a common docking site, thereby suppressing STAT1 tyrosine phosphorylation. 2) Retention of STAT1 in the cytoplasm secondary to competition for limiting nuclear import factors or interaction with excess cytoplasmic STAT3. 3) Sequestration of STAT1 in STAT3:STAT1 heterodimers resulting in the diminished formation of transcriptionally active STAT1 homodimers. These results would explain diminished activation of CXCL9 and CXCL10 by IFNα in

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FIGURE 7. STAT3 sequesters STAT1 into STAT1:STAT3 heterodimers and reduces IFNα induction of DNA binding by STAT1 homodimers. A, control and STAT3hi THP-1 cells were stimulated with 10,000 units/ml of IFNα for 15 min, and the whole cell and nuclear extracts (5 μg) were subjected to EMSA with [32P]hSIE oligonucleotide probe. B, the same IFNα-stimulated samples from A were preincubated for 15 min at 4 °C with control or STAT3-specific antiserum as previously described (32). C and D, THP-1 cells expressing high levels of both STAT3 and STAT1 (STAT3hi STAT1hi) were generated, stimulated with IFNα, and nuclear extracts were subjected to EMSA and supershift analysis as in A and B. E, cells were stimulated with 100 units/ml of IFNγ for 15 min, and nuclear extracts (5 μg) were analyzed by EMSA with a [32P]ISRE oligonucleotide probe. F, cells were stimulated with 10,000 units/ml of IFNα for 15 min, and the nuclear extracts (5 μg) were analyzed by EMSA with [32P]ISRE oligonucleotide probe.

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STAT3β1 cells if STAT1:STAT3 heterodimers are less effective in activating these gene promoters than are STAT1 homodimers. Although formation of STAT1:STAT3 heterodimers was first observed in 1994 (56), these heterodimers are always induced together with STAT1 or STAT3 homodimers, or both, and determination of heterodimer function has not been experimentally tractable. Our studies are consistent with the notion that heterodimerization with STAT3 can attenuate STAT1 transcriptional activity on inflammatory gene promoters, but further direct evidence to confirm this idea awaits the development of experimental systems where the function of these heterodimers can be studied.

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