Do the NOD2 and TLR4 bacterial receptor proteins have differing transcription factor dimer preferences, and can this preference account for the observed inhibitory interaction between these two proteins?

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Abstract

Inhibitory interactions between the NOD2 and TLR4 bacterial receptors plays an important role in the maintenance of intestinal homeostasis. Dysregulation of the NOD2 inhibitory pathway has been highly associated with the inflammatory autoimmune disease Crohn's Disease. However, the mechanism behind NOD2's inhibitory influence on TLR4 are unclear. In this paper, I suggest that this interaction stems from NOD2's preference for IRF4 producing NF-kB. Western Blot and ELISA analyses were performed to determine if NOD2 has an aversion to the RELA inflammatory subunit, if TLR4 has a preference for this protein, and if these differing preferences could account for NOD2 and TLR4's interaction. It was found that NOD2 stimulation caused a lower concentration of RELA to translocate into the nucleus that TLR4 stimulation. This data concludes that RELA is preferentially activated by TLR4. Further research is required to determine if other commonly inflammatory REL subunits are also prefered by TLR4, and if anti-inflammatory subunits such as p50 are prefered by NOD2.

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Introduction

NOD2 and Crohn's Disease:

Crohn's Disease (CD) is a chronic inflammatory autoimmune disorder which affects more than 1 in 200 people in developed countries (Cleynen, 156). CD manifests as intestinal inflammation, recurring ulceration, exaggerated response to normal intestinal bacteria, elevated levels of intestinal bacteria, and most importantly, overactivity of the nuclear factor kappa B (NF-kB) stress response transcription factor (Strober et.al, 249; Cleynen, 156).

Crohn's Disease has a complex etiology. While genetic mutations have been linked to the development of the Crohn's disease, evidence also implicates microbiome compositions, environmental factors, and exposure to bacteria as a child as factors of pathogenesis(Lee, 1549; Nuñez). No given combination of these factors have been shown to give a 100% chance of pathogenesis. To develop better treatment methods for CD, cellular pathways commonly mutated in CD patients have been under intense study.

Mutations in over 163 different proteins have been found to contribute to CD development. Genome wide association studies have determined multiple genetic risk loci for CD in the pericentromeric region of chromosome 16. The Nucleotide-binding and Oligomerization Domain containing protein #2(NOD2) gene lies in the center of this risk region. Different mutations have been shown to determine localization of the disease and severity of symptoms (Palmieri; Cleynen,156). Mutations in the NOD2 gene have been associated with severe symptoms, as well as disease localization to the ileum and small intestine (Nuñez, 603; Sahbatou, 599).

Two missense and one nonsense single nucleotide polymorphisms(SNPs) in NOD2- 3020ins C, 2722G->C, and 2104C->T- have been shown to greatly increase the risk of developing CD (Lee, 1549). The 3020ins C nonsense mutation is particularly dangerous,

conveying a 17 fold likelihood of CD pathogenesis (Nuñez, 603).

NOD2 Signaling Pathway:

NOD2 is a pathogen associated molecular pattern (PAMP) recognition protein which affects inflammatory gene transcription through the nuclear factor kappa B (NF-kB) transcription factor. NOD2 consists of 2 N-terminal caspase recruitment domains, a central NACHT domain, and 10 leucine rich repeats(LRR) on its C-terminal (Boyle, 140178). NOD2's primary ligand is the bacterial cell wall component muramyl dipeptide (MDP). NOD2 has also been shown to interact with as many as 30 other proteins, and is essential for the targeting of autophagic machinery to bacterial entry sites. NOD2 is highly expressed in mononuclear cells, and intestinal epithelial cells (Boyle, 140178; Strober, 249).

NOD2 remains in an autoinhibited state via interaction between its NACHT and LRR regions, until it detects MDP from phagocytosed or invasive bacteria. Upon ligation, NOD2 forms a complex with receptor interacting protein 2(RIP2). The formation of a NOD2:RIP2 protein complex has been shown to be essential to NOD2 signaling, as RIP2 deficient cells fail to respond to MDP (Jeong, 269; Strober, 249).

The NOD2:RIP2 complex undergoes a post-translational modification called polyubiquitination. Polyubiquitination is the addition of several ubiquitin protein chains to a target protein. This process signals for protein activation, and also acts as a cofactor to the TGF-β- activated kinase 1 functional complex (TAK1:TAB).

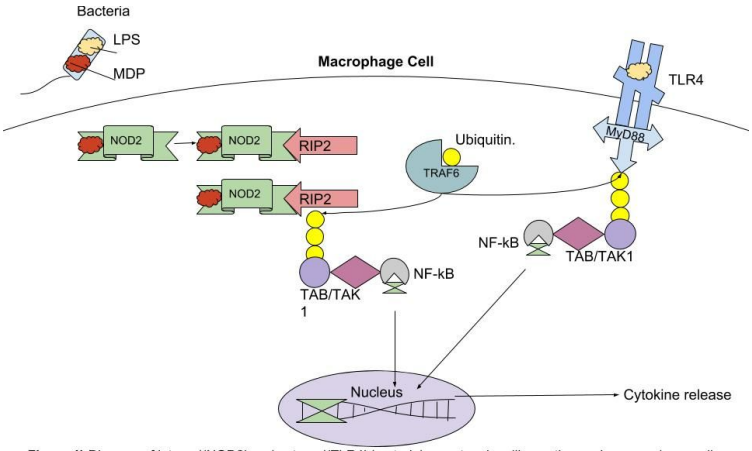

Figure 1) Diagram of internal(NOD2) and external(TLR4) bacterial receptor signalling pathways in macrophage cells. When NOD2 detects the bacterial cell wall compound MDP, NOD2 recruits the RIP2 scaffolding protein. RIP2 is then polyubiquitylated by E3 ubiquitin ligases such as TRAF6, and a TAB/TAK1 kinase complex is bound to the newly formed ubiquitin chain. TAK1 then phosphorylates the inhibitor of nuclear factor-kB (IkB) protein complex. This releases a dimer of NF-kB subunits which enters the nucleus and causes the excretion of stress response cytokines. TLR4 activates NF-kB in a very similar manner, by first ubiquitinating its MyD88 interaction domain, and then phosphorylating the IkB protein complex, releasing NF-kB.

Polyubiquitinated RIP2:NOD2 then translocates to the site of bacterial entry in the plasma membrane, and transfers its polyubiquitin chains and TAK1/TAB complex

to the inhibitory units of NF-kB(IkB), thus phosphorylating its IkB and activating NF-kB dimers which are floating in the cytoplasm (Boyle, 140178; Abbott, 6012).(Figure 1)

NOD2 activation is biphasic. Upon activation NOD2 induces the secretion of pro-inflammatory cytokines, but then prevents inflammation caused by stimulation of other NF-kB signaling proteins later on (Watanabe, 800).

NOD2 plays many roles in intestinal homeostasis, and is highly expressed in both pddaneth intestinal epithelial cells and intestinal macrophage immune cells. Because of its varied functions, it is unclear which homeostatic properties of NOD2 are impacted by CD associated mutations in the gene (Strober, 249; Boyle, 140178).

Antibacterial Peptide Secretion:

There are two emerging theories explaining how NOD2 dysfunction affects the development of Crohn's Disease. The dominant of the two theory suggests that NOD2 mutation impairs the production of antibacterial peptides.

NOD2 plays a critical role in the expression of important antibacterial proteins called defensins by Paneth intestinal epithelial cells. Defensins maintain the balance of bacteria in the intestinal mucosa, and are mainly produced in intestinal crypts of the ileum and ascending colon- the CD disease location associated with NOD2 dysfunction. NOD2 mutation has been shown to decrease the transcription of alpha-defensins 5 and 6 (Strober, 249).

This theory suggests that NOD2 mutation plays a passive role in immune dysfunction. Instead of impairing cells' ability to fight bacteria, the decrease in defensin production by paneth cells allows bacteria to grow unchecked, and overwhelm normal barrier functions. Normally non-pathogenic bacteria are then able to permeate the intestinal walls, and cause the deep tissue inflammation observed in CD (Hedl, 231).

This theory of pathogenesis is promising, a as it directly associates the overgrown microbiome seen in CD with dysfunction of NOD2. However, it has been shown that decrease in defensin production is not a primary disease factor. Decrease in defensin excretion by paneth cells in CD patients is not sufficient to cause the observed levels of inflammation in CD. Only a single NOD2 polymorphism, L1007insC, has been shown to significantly decrease defensin production (Strober, 249). This suggests that lack of defensin production is a symptom, not a cause, of NOD2 malfunction.

NOD2 Coordination of Innate Immune Responses:

A new and promising model relating NOD2 dysfunction to Crohn's disease stems from NOD2's ability to inhibit inflammation caused by other PAMP receptors, such as Toll Like Receptors(TLR) 2 and 4. This theory suggests that NOD2 activation keeps inflammation caused by other bacterial receptors in check, possibly through regulatory cycles in the NF-kB transcription factor.

NOD2 is used for several processes by intestinal macrophages, namely the targeting of phagocytic proteins to bacterial entry sites, the recognition of MDP, and subsequent activation of NF-kB(Boyle, 140178). Pre-stimulation of NOD2 has been shown to downregulate inflammation responses caused by TLRs. (Watanabe, 800; Strober, 249; Liew, 446).

TLRs are peripheral bacterial detection proteins which signal through the NF-kB pathway, similar to NOD2. TLR activation causes an increase in secretion of pro-inflammatory cytokines upregulated by NF-kB, such as the inflammatory cytokine Tumor Necrosis Factor alpha (TNF-alpha). (Strober, 249; Liew, 446).

TLRs are tightly regulated by several molecules in the cell. One protein which dampens TLR response is called interferon regulatory factor 4(IRF4). IRF4 competes for necessary binding sites in TLRs' MyD88 amino group. The preferential binding of IRF4 to

MyD88 prevents TLRs from being polyubiquitinated, and thus prevents TLRs from activating NF-kB inflammation (Liew, 446; Strober). The IRF4 gene can be activated by a certain NF-kB subunit, p50, thus causing a self regulatory cycle in NF-kB activation (Strober, 249). CD related polymorphisms prevent NOD2 from activating the NF-kB signaling pathway, and thus prevent the induction of TLR inhibiting proteins such as IRF4. Thus, the modern theory of NOD2 dysfunction and CD pathogenesis suggests that mutated NOD2 does not activate NF-kB proteins which dampen inflammatory TLR signaling.

This modern theory of NOD2 dependent CD pathogenesis is very promising, as it is directly correlated with the overactivity of NF-kB commonly seen in CD and explains why TNF-alpha suppressing medication is effective at treating CD. However, TLR activated NF-kB does not show the anti-inflammatory properties of NOD2 activated NF-kB.

In order for this alternate pathway of CD pathogenesis to remain a viable option for treatment development, an explanation for the different results of NOD2 and TLR activated NF-kB must be suggested (Liew, 446; Strober, 249).

As both the TLR4 and NOD2 signaling pathways overlap at the NF-kB transcription factor (figure 1), I suggest that the inhibitory effect of MDP prestimulation on inflammatory cytokine release is due to a preferential activation of IRF4 enhancing NF-kB dimers by NOD2, and preferential activation of pro-inflammatory NF-kB dimers by TLR4.

Nuclear Factor kB:

NF-kB is a transcription factor responsible for the majority of stress responses in the cell (Mercurio, 860). Over 150 different stimuli activate the NF-kB pathway, and there are subsequently over 150 NF-kB promoter sites in the genome (Mercurio, 860). The functional NF-kB protein consists of two subunit proteins bound together, called a dimer (figure 2).

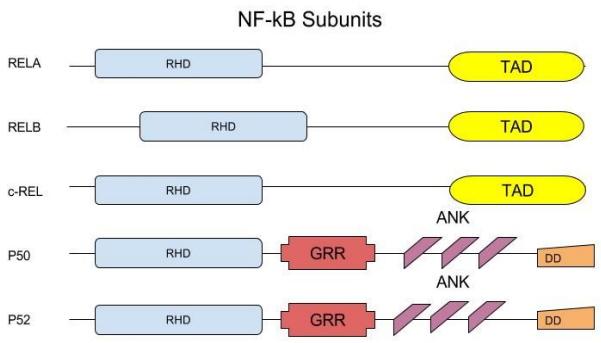

Figure 2) Protein topology of the five Nuclear Factor-kB (NF-kB) subunits. While all subunits share the REL homology domain(RHD), only RELA, RELB, and c-REL contain transcription activation domains(TAD). This means that while all combinations of subunits affect DNA transcription, only the REL family of subunits can directly activate genes. The p50 and p52 subunits contain glycine rich regions(GRR) as well as ankyrin repeats(ANK) and a death domain(DD) allowing them to repress gene activity and play a role in mediating inflammatory chemical release.

Which genes NF-kB dimers bind to, and how the subunit dimers affect transcription is dependent on the combination of subunits and the activating stimuli.

There are five NF-kB subunits, which are divided into two categories: REL proteins and NF-kB proteins. REL proteins include RELA, RELB, and c-REL. These subunits differ from the NF-kB family of subunits in their ability to directly influence gene transcription (Cao, 26041). REL proteins contain a transcriptional activation domain on their C-termini. This allows for REL subunits to bind to DNA, and directly activate inflammatory gene transcription such as TNF-alpha (Loop, 1; Lim).

NF-kB subunit proteins include p50, and p52. These subunits contain ankyrin repeats as well as death domains on their C-termini. These regions allow for p50 and p52 to down-regulate inflammatory gene expression when bound to REL proteins (Loop, 1; Mercurio, 860). NF-kB subunits are also associated with increased production of anti-inflammatory cytokines such as IL-10 and IRF4.(Cao, 26041).

NF-kB subunit proteins can form over 20 different dimers, each with differing DNA binding sites and differing transcriptional activity. However, all of these dimer combinations share a common inhibitory protein complex(IkB). IkB retains NF-kB dimers in the cytoplasm of cells by blocking the nuclear localization signals in the subunits' RHD domain, until NF-kB is needed (Mercurio, 860) .

In order to activate NF-kB, a polyubiquitin chain and a ubiquitin dependent TAK1/TAB signaling complex must be bound to IkB. TAK1/TAB then phosphorylate the IkB complex and marks it for degradation in the proteasome (Abbott, 6012).

Interestingly, precursor proteins for p50 and p52 are contained in the IkB protein, and degradation of IkB releases functional p50 and p52 subunits. This finding suggests that NF-kB activation creates a self-regulatory loop to check inflammation, as p50 has been shown to be the only NF-kB subunit to upregulate the powerful anti-inflammatory cytokine IL-10 and the TLR inhibiting protein IRF4(Mercurio, 860; Loop, 1; Cao, 26041).

The combined details of these findings suggest that P50 containing NF-kB dimers and c-REL containing NF-kB dimers are preferentially activated by NOD2 and TLRs respectively, in order to maintain a self-regulatory NF-kB loop. To test this theory, the comparative concentrations of NF-kB subunits translocated to the nucleus after stimulation with MDP or LPS will be investigated.

Methods

In order to determine whether or not NOD2 and TLR4 have preferences for pro or anti-inflammatory NF-kB subunits, macrophages will be stimulated with either LPS(TLR4 ligand) or MDP(NOD2 ligand), and their nuclei will then be extracted. The concentrations of specific NF-kB subunits moved into the nuclei of LPS or MDP stimulated cells will then be compared using a western blot technique. I will also compare levels of TNF-alpha secreted by cells to establish an "index" for NF-kB activity.

If my hypothesis is correct, I expect to find higher concentrations of REL subunits in LPS stimulated nuclei, and higher concentrations of p50 and p52 in MDP stimulated nuclei

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Cell Stimulation

RAW 264.7 cells were stimulated with 0.1, 1, or 10 ug/mL of lipopolysaccharide (LPS) or muramyl dipeptide(MDP) for 24 and 48

hours. Using Enzyme Linked Immunosorbent Assay(ELISA) purchased from Thermofisher Scientific, it was determined that 1 ug/mL of both MDP and LPS should be used for my determination of transcription factor preferences, and media samples should be taken at time points of 1 hour , 3 hours , and 6 hours for NF-kB activity indexes.

ELISA

Enzyme linked immunosorbent assays(ELISA) for tumor necrosis factor alpha were performed after 24 and 48 hours of MDP and LPS stimulation in order to determine the proper concentrations of MDP and LPS to use for nuclear translocation analysis, and to act as an index for NF-kB activity.

ELISA analysis were also performed on media samples taken from cells stimulated for 1, 3, and 6 hours along with cell samples, to correlate concentrations of secreted tumor necrosis factor alpha with concentrations of translocated NF-kB subunits.

Nuclear Isolation

Nuclei of cells stimulated with MDP, LPS, or both for 1, 3, and 6 hours were isolated using nuclear extraction reagents purchased from Thermofisher Scientific.

Western Blotting

Proteins from isolated nuclei were separated by size through polyacrylamide gel electrophoresis, and then transferred onto a nitrocellulose membrane. The membrane was then blocked, and washed in primary and HRP conjugated secondary antibody solutions. Membranes were coated in HRP substrate, and observed under x-ray light.

Results

Two ELISAs were run for this experiment, one to determine which concentration of stimuli would be best for cell stimulation, and another ELISA experiment linked to western blot data. While LPS stimulation showed fairly little variation between 24 and 48 hour samples, MDP stimulation caused over double the amount of TNF-alpha released in 24 hours to be released over 48 hours (Chart 1).

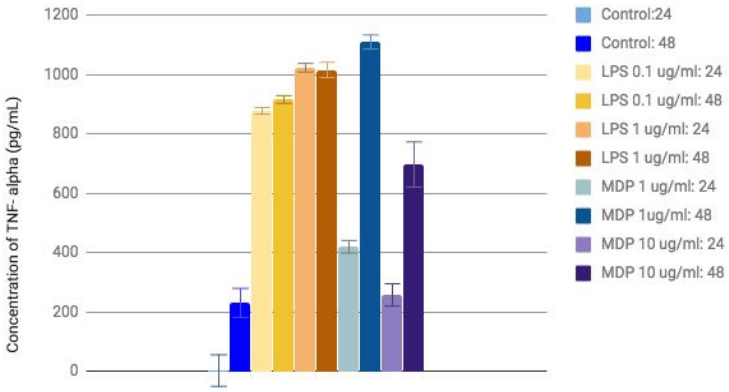

Chart 1) Concentrations of TNF-alpha in media 24 and 48 hours after stimulation. RAW264.7 cells were stimulated with either 0.1 or 1 ug/mL of LPS, 1 or 10 ug/mL of MDP, or nothing. Media samples were then collected after 24 and 48 hours. TNF-alpha concentration was determined using an ELISA.

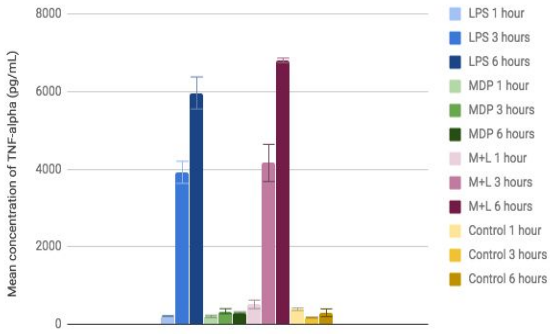

Chart 2) Concentrations of TNF-alpha in media 1, 3, and 6 hours after stimulation. RAW264.7 cells were stimulated with 1 ug/mL of LPS, MDP, both or neither, and media samples were collected in conjunction with cell samples after 1, 3, and 6 hours.

Western Blot analysis showed that more RELA translocated into the nucleus of cells stimulated with LPS than those stimulated with MDP. Cells stimulated with both MDP and LPS showed a similar concentration of RELA in the nucleus as that of LPS alone(figure 3), and similar amounts of secreted TNF-alpha(Chart 2). RELA concentration increased over time for MDP, but decreased slightly over time for both LPS+MDP, and LPS alone.

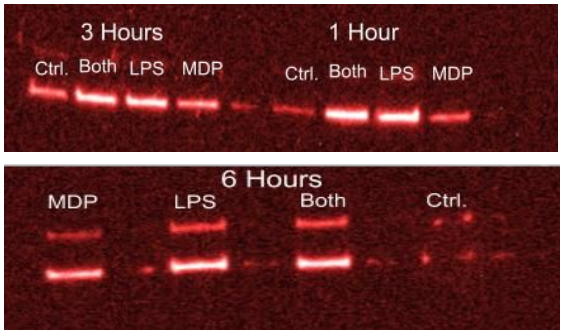

Figure 3) Western blot analysis of RELA concentrations in stimulated nuclei. Nuclei were isolated from cells stimulated as described in chart 2.

TNF-alpha ELISA data shows that while the concentration for RELA in the nuclei of cells stimulated with LPS alone and MDP with LPS decreased slightly over time, TNF-alpha steadily increased over time (Chart 2)(Figure 3).

Discussion

A novel theory relating dysfunction of NOD2:TLR4 inhibitory interactions to NOD2's role in the development of Crohn's Disease suggests that activation of the NOD2 pathway is essential to mediating NF-kB linked inflammation. While the inhibitory interaction between the NOD2 and TLR4 has been established, the biochemical mechanism for this interaction remains unclear.

Both NOD2 and TLR4 affect gene transcription through the NF-kB transcription factor. NF-kB is a dimer of five possible subunits: RELA, c-REL, RELB, p52, and p50. While c-REL and RELA have been shown to upregulate the production of pro-inflammatory cytokines, such as TNF-alpha and IL-1B, p50 has been shown to upregulate expression of anti-inflammatory cytokines such as IL-10 and the TLR4 inhibiting protein IRF4. The distinct roles of these NF-kB subunits suggests that the differentiation of NOD2 and TLR4 signaling may occur due to preferences for certain NF-kB dimer combinations.

RELA and c-REL have been experimentally shown to cause the upregulation of TNF-alpha secretion. This relationship allowed the linking of ELISA analysis of secreted TNF-alpha and Western Blot analysis of nuclear RELA concentration.

Cells stimulated with the NOD2 ligand MDP showed less translocation of RELA into

their nucleus than cells stimulated with LPS and MDP or LPS alone (figure 3). This data suggests that TLR4 has a higher affinity for RELA containing NF-kB dimers than NOD2. This data is bolstered by the linked ELISA analysis of TNF-alpha cytokine concentration in media (Chart 2). The observed upregulation of TNF-alpha after TLR4 stimulation is consistent with the theory suggesting a TLR4 preference for pro-inflammatory NF-kB dimer preference.

NOD2 stimulation showed very low levels of TNF-alpha production immediately after stimulation. This data in combination with the Western Blot analysis data for RELA suggest that NOD2 shows a much lower affinity for- if not an aversion to- REL containing NF-kB (Chart 2) (Figure 3).

While this data supports the theory of NOD2 inhibition of TLR4 response for RELA alone, more data is needed to bolster this observation. Western Blot analysis of c-REL, p50 and p52 should be conducted to support my preliminary analysis.

Conclusion

In this study, it was found that costimulation of MDP and LPS causes an increase in inflammatory cytokine secretion. NOD2 is suggested to have two phases of activation- first enhancing inflammatory cytokine expression, then inhibiting it upon later TLR4 stimulation. This theory of NOD2 function is controversial. Future studies should adjust the methods by which cells are stimulated to account for this 2 phase activation process. Cells should be stimulated with MDP 6-18 hours before stimulation with LPS, to determine if prestimulation “cross-tolerizes” cells to LPS.

Through linked western blot and ELISA analysis, it was found that TLR4 has a higher affinity for the inflammatory NF-kB subunit RELA than NOD2. This data suggests that TLR4 linked activation of NF-kB has a preference for REL subunits, thus supporting my hypothesis on the inhibitory relationship between NOD2 and TLR4.

RELA is an activator of the TNF-alpha gene, and other proinflammatory cytokines. TLR4 stimulation leads to a higher rate of RELA translocation than NOD2 stimulation, however it is unknown whether this preference for RELA is sufficient in ascribing the differences in inflammatory response between NOD2 and TLR4. It is also unclear if this preference can account for the modern theory of NOD2 related CD pathogenesis involving impaired autoimmune regulation.

Further analysis of the NF-kB subunits c-REL and p50 will be instrumental in supporting the suggested TLR4 preference for proinflammatory cytokines. If TLR4 is found to have a similar preference for c-REL, and an aversion to p50, it is likely that the proposed idea of transcription factor preferences will be correct.

Similarly, establishing a NOD2 preference for the anti-inflammatory subunit p50, and confirming its suggested aversion to REL subunits will be instrumental to further research supporting the transcription factor preference model of NOD2 inhibition of TLR4.

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