

Mechanisms of interleukin-10-mediated immune suppression

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SUMMARY

Specific immune suppression and induction of anergy are essential processes in the regulation and circumvention of immune defence. Interleukin-10 (IL-10), a suppressor cytokine of T-cell proliferative and cytokine responses, plays a key regulatory role in tolerizing exogenous antigens during specific immunotherapy (SIT) of allergy and natural exposure to antigens. Specific T-cell tolerance is directed against the T-cell epitopes of an antigen and characterized by suppressed proliferative and T helper type 1 (Th1) and type 2 (Th2) cytokine responses. IL-10 elicits tolerance in T cells by selective inhibition of the CD28 co-stimulatory pathway and thereby controls suppression and development of antigen-specific immunity. IL-10 only inhibits T cells stimulated by low numbers of triggered T-cell receptors and which therefore depend on CD28 co-stimulation. T cells receiving a strong signal from the T-cell receptor alone, and thus not requiring CD28 co-stimulation, are not affected by IL-10. IL-10 inhibits CD28 tyrosine phosphorylation, the initial step of the CD28 signalling pathway, and consequently the phosphatidylinositol 3-kinase p85 binding to CD28. Together these results demonstrate that IL-10-induced selective inhibition of the CD28 co-stimulatory pathway acts as a decisive mechanism in determining whether a T cell will contribute to an immune response or become anergic.

INTRODUCTION

Specific activation of T cells requires stimulation through the T-cell receptor (TCR) and a co-stimulatory signal, generated by the engagement of multiple cell surface receptors with their ligands.^{1,2} A major co-stimulatory signal is delivered to the T cells by the interaction of CD28 with molecules of the B7 family (CD80, CD86) displayed by antigen-presenting cells (APC).^{3,4} TCR stimulation without co-stimulatory signals induces tolerance or anergy in T cells.^{5–8} Anergy represents a state of immune inactivation characterized by abolished proliferative and cytokine responses. It is actively generated by a number of molecular events and can be reversed by certain cytokines.^{2,7–11} Although the molecular mechanisms have not been elucidated so far, specific T-cell anergy induced by the autocrine action of interleukin-10 (IL-10) has been demonstrated during natural exposure to antigens, during specific immunotherapy and various diseases in human and mice.^{11–16} The co-stimulatory signal induced by complexing CD28 with specific monoclonal antibodies (mAbs) or by interaction with

B7 counter-receptors enhances the antigen-dependent T-cell proliferation and cytokine production.^{4–6} In contrast, anergy is elicited in T cells by blocking the CD28/B7-mediated cellular interaction, as shown *in vitro* and *in vivo*.^{5,6}

Allergic diseases are basically immunological disorders related to the development of distinct T-cell cytokine patterns, including increased secretion of allergic inflammatory cytokines, in particular of IL-4, IL-5 and/or IL-13. Whereas the symptoms of immediate- and late-type allergic reactions can be ameliorated by various pharmacological treatments, the allergen-specific immunotherapy (SIT) represents the only curative approach for specific Type I allergy. Although SIT is most efficiently used in allergy to insect venoms and allergic rhinitis, the mechanism by which SIT achieves clinical improvement remained unclear until recently. A rise in allergen-blocking immunoglobulin G (IgG) antibodies, particularly of the IgG4 class,¹⁷ the generation of IgE-modulating CD8⁺ T cells and a reduction in numbers of mast cells and eosinophils, including the release of mediators,^{18–20} were shown to be associated with successful SIT. Furthermore, SIT was found to be associated with a decrease in IL-4 and IL-5 production by CD4⁺ T cells, and in some cases with a shift towards increased interferon- γ (IFN- γ) production.^{11–13,21–26} However, recent studies demonstrated that the induction of an anergic state in peripheral T cells and recovery from anergy by cytokines from the tissue microenvironment are essential steps in the mechanism of SIT^{11–13} (Fig. 1). Thus, conditions of the immunological microenvironment and production of cytokines by cells from the tissue may determine

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Abbreviations: BV, bee venom; PIT, peptide immunotherapy; PLA, phospholipase A₂; PI3-K, phosphatidylinositol 3-kinase; SEB, Staphylococcal enterotoxin B; SIT, specific immunotherapy; TCR, T-cell receptor.

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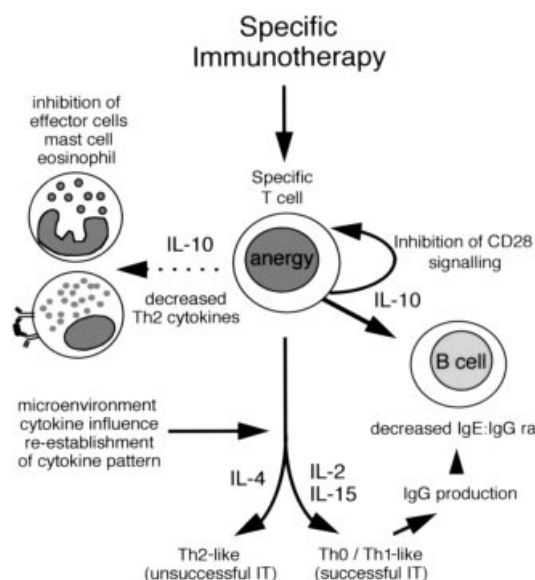


Figure 1. Immunological mechanisms of SIT. Continuous treatment with allergen establishes a state of peripheral anergy in specific T cells, which is characterized by blocked CD28 signalling and suppressed proliferative and T-cell cytokine responses and simultaneous increase in IL-10 production. In consequence, activation, priming and survival of allergic inflammatory effector cells are down-regulated. The anergic T cells can be recovered by cytokines from the tissue microenvironment. In successful SIT, anergic T cells recover by the influence of IL-2 and/or IL-15 to produce Th0/Th1 cytokines. In an atopic or polyallergic microenvironment, IL-4 reconstitutes a Th2 cytokine pattern and may reactivate allergic responses.

whether SIT develops towards a successful or unsuccessful treatment.

An especially suitable model for the study of human cellular and molecular mechanisms, regulating specific allergy and normal immunity, is provided by the immune response to bee venom (BV)^{11,12,21,22,27–29} (reviewed in refs 13,30–32). The BV phospholipase A₂ (PLA) represents the major antigen and allergen of BV, and SIT with whole BV (BV-SIT) or with short PLA peptides representing immunodominant T-cell epitopes (peptide immunotherapy; PLA-PIT) was applied successfully. In addition, natural exposure to multiple bee stings in non-allergic bee keepers enabled the study of tolerance of high doses of allergen exposure in healthy individuals.^{12,33}

ESSENTIAL STEPS IN SIT: PERIPHERAL T-CELL TOLERANCE AND REACTIVATION BY MICROENVIRONMENTAL CYTOKINES

The immunological mechanism of SIT was investigated in BV-SIT^{11,12} and further elucidated in PLA-PIT²² using a mixture of three peptides representing the immunodominant T-cell epitopes PLA45–62, PLA82–92 and PLA113–124. In both BV-SIT and PLA-PIT, successfully treated patients developed specific T-cell unresponsiveness against the entire PLA allergen as well as the three T-cell epitope-containing peptides. After 60 days of treatment, the specific proliferative T-cell response and secretion of T helper type 2 (Th2) cytokines, IL-4, IL-5 and IL-13, as well as the Th1 cytokines IL-2 and IFN- γ were suppressed (Fig. 1). The protein purified derivative of

M. tuberculosis (PPD) or tetanus toxoid (TT) control responses were not affected by these treatments, indicating that the suppressive effect of SIT and PIT is specific to the allergen.

The induction of an anergic state in Th2 cells represents an active process, associated with increased levels of basal tyrosine kinase activity, the cytokine production and CD25 up-regulation. It appears to be related to alterations in the signalling pathways mediated through the TCR. The anergized Th2 cells failed to respond to anti-CD3 stimulation with increased tyrosine phosphorylation of p56^{lck} and ZAP70 kinases. In addition, intracellular calcium flux, observed in untreated Th2 cells in response to anti-CD3 mAb, was absent in anergic Th2 cells.⁷

The abrogated proliferative response was fully recovered by stimulation of anergic T cells in the presence of antigen and IL-2 or IL-15 (Fig. 1). Also, the full capacity for IL-2 and IFN- γ secretion was re-established by this cytokine treatment. In contrast, specific stimulation in the presence of IL-4 induced IL-4, IL-5 and IL-13 secretion and therefore, recovered a Th2 cytokine pattern typical for an allergy. Thus, microenvironmental tissue cytokines recover and regulate T cells from SIT-induced anergy.^{11,13} They can generate distinct Th0/Th1 cytokine patterns associated with successful therapy and normal immunity, or reactivate Th2 cells supporting the persistence of the allergic response (Fig. 1). In this respect, successful SIT may be more difficult to achieve in an established polyspecific allergy and atopy, and treatment has to be applied at an early stage of disease.

Decreased T-cell proliferative responses in SIT were demonstrated also in allergy to ragweed, cat dander, grass pollen and BV.^{34–36} In mice, antigenic peptides of house dust mite and cat allergen were shown to induce anergy in T cells,^{37,38} and studies with T-cell peptides of *Fel d 1*, clearly indicated peripheral tolerance induction in T cells by PIT of cat allergy.^{25,39} In a recent study, application of high doses of T-cell epitope peptides of cat allergen were shown to initiate a T-cell-dependent late asthmatic reaction, without the requirement for an early IgE/mast cell-dependent response, in sensitized asthmatic subjects.⁴⁰

DISTINCT SPECIFIC T-CELL RESPONSE PROFILE DURING SIT AND NATURAL HIGH DOSE OF ANTIGEN EXPOSURE

High IL-10 production and decreased proliferation and Th1 and Th2 cytokines

The anergized cells showed suppressed PLA-specific T-cell proliferative and cytokine responses that could be reconstituted by *ex vivo* neutralization of endogenous IL-10. This indicates that IL-10 is actively involved in the development of anergy in specific T cells. Furthermore, whereas in both BV-SIT and PLA-PIT the antigen and peptide-induced proliferative responses and Th1 and Th2 cytokine production decreased, the IL-10 production simultaneously increased and reached maximal levels after 4 weeks, when the specific anergy was fully established. The cellular origin of IL-10 was demonstrated by intracytoplasmic IL-10 staining in peripheral blood mononuclear cells (PBMC) and co-expression of cellular surface markers.¹² Intracellular IL-10 significantly increased after 7 days of SIT in the antigen-specific T-cell population and

activated CD4⁺ T lymphocytes. After 4 weeks of SIT intracytoplasmic IL-10 was also increased in monocytes and B cells, suggesting an autocrine action of T-cell-secreted IL-10 as a pivotal step in the induction phase of T-cell anergy and its maintenance by IL-10-producing APC and non-specific bystander T cells.¹²

Interestingly, the same features of anergy were found in the T cells of healthy bee keepers, who had previously been stung by high numbers of bees. These naturally anergized individuals show increased numbers of IL-10-producing CD4⁺ CD25⁺ T cells and monocytes similar to allergic patients after BV-SIT. Neutralization of endogenous IL-10 in PBMC cultures from these individuals fully reconstituted the proliferative T-cell response and cytokine production. Beside SIT and PIT of BV allergy, evidence for induction of peripheral T-cell anergy was recently obtained in SIT of wasp venom allergy, grass pollen asthma, conjunctivitis and rhinitis.^{24,35,36,41,42} Furthermore, down-regulated T-cell responses were reported in PIT of cat allergy.²⁵ Moreover, a SIT-induced IL-10 increase was demonstrated also in wasp allergy, grass pollen asthma and nasal immunotherapy of weed-induced allergic rhinitis.^{24,41,42}

IL-10 is a major regulatory cytokine of inflammatory responses. It was originally described as a mouse Th2 cell factor, inhibiting cytokine synthesis by Th1 cells.⁴³ However, increasing evidence accumulated that IL-10 acts as a general inhibitor of proliferative and cytokine responses of both Th1 and Th2 cells *in vitro* and *in vivo*.^{44–46} IL-10 is released by mononuclear phagocytes,^{44,45} natural killer cells and by both Th1 and Th2 type lymphocytes.⁴⁶ In mice, IL-10 administration before allergen treatment induced antigen-specific T-cell tolerance and demonstrated the pivotal role of IL-10 in establishment of peripheral T-cell anergy.¹⁴ Moreover, inhibition of graft-versus-host disease by IL-10 and allograft rejection in human leucocyte antigen-mismatched, bone-marrow-transplanted severe combined immunodeficient patients gives further evidence for a key role of this cytokine in the induction and maintenance of an anergic state.¹⁵ Similarly, inappropriate stimulation of tumour-reactive human T cells was shown to result from increased endogenous IL-10 production by these cells,¹⁶ indicating also a role for IL-10 in tumour-specific anergy. Recently, IL-10-derived regulatory CD4⁺ T cells producing IL-10 but not IL-2 and IL-4, which suppressed antigen-specific T-cell response *in vitro* and prevented antigen-induced murine colitis, were identified in humans and in mice.⁴⁷ Apparently, T cells observed during SIT and natural antigen exposure represent the so-called T-regulatory 1 cells in humans.

Regulatory role of IL-10 on IgE production and effector cells of allergy

The serum levels of specific IgE and IgG4 antibodies delineate allergic and normal immunity to allergen. Whereas peripheral anergy was demonstrated in specific T cells, the capacity by B cells to produce specific IgE and IgG4 antibodies was not abolished. In fact, specific serum levels of both isotypes increased during the early phase of treatment. However, the increase in specific IgG4 was more pronounced and the ratio of specific IgE to IgG4 decreased by 10–100-fold. Also the *in vitro* production of PLA-specific IgE and IgG4 antibodies by PBMC changed in parallel to the serum levels of specific isotypes. A similar change in specific isotype ratio was observed in SIT

of various allergies. Moreover, IL-10 that was induced and increasingly secreted by SIT, appears to counter-regulate antigen-specific IgE and IgG4 antibody synthesis. It is a potent suppressor of both total and allergen-specific IgE, while simultaneously IgG4 formation is increased.^{12,13} Thus, IL-10 not only generates anergy in T cells; it also regulates specific isotype formation and skews the specific response from an IgE-dominated to an IgG4-dominated phenotype.

Despite the fact that a definite decrease in IgE antibody levels and IgE-mediated skin sensitivity normally requires several years of treatment, most patients are protected against bee stings already at an early stage of BV-SIT. Increase of allergen-specific IgG4 antibodies, blocking IgE binding to the allergen, may explain only the late phase of protection by SIT. At the early phase of SIT however, a decrease in histamine and sulphidoleukotriene release from basophils, may be of more relevance. This decreased basophilic mediator releasability⁴⁸ can be attributed to suppression of cytokines in anergic T cells. There is clear evidence that effector cells of the allergic inflammation (mast cells, basophils and eosinophils) require T-cell cytokines for priming, survival and activity.^{49,50} In addition, IL-10 was shown to reduce TNF- α GM-CSF and IL-6 generation from mouse bone marrow and rat peritoneal mast cells.^{51,52} Moreover, IL-10 down-regulates eosinophil function and activity and suppresses IL-5 production by human resting Th0 and Th2 clones.^{53,54} Furthermore, IL-10 inhibits endogenous granulocyte-macrophage colony-stimulating factor (GM-CSF) production and CD40 expression by activated eosinophils and enhances eosinophil cell death.^{55,56}

A MOLECULAR BASIS FOR DIRECT T-CELL SUPPRESSION BY IL-10

IL-10 inhibits T cells only if co-stimulation is required

The molecular mechanisms of T-cell suppression by IL-10 was investigated in antigen-specific PBMC cultures, purified CD45RO⁺ T cells and T-cell clones. IL-10 inhibited the proliferative T-cell response in PBMC to various antigens, and the superantigen staphylococcal enterotoxin B.³³ However, IL-10 did not affect the proliferative responses of T cells that were stimulated by anti-CD3. In contrast, IL-10 significantly inhibited the anti-CD28-stimulated proliferation. The analysis of TCR numbers on T cells demonstrated the essential requirement for co-stimulation in T-cell activation and its relation to the number of triggered TCRs.³³ IL-10 inhibited the T-cell proliferation within a certain range of triggered TCRs that T cells require for co-stimulation. T cells which were stimulated by different concentrations of anti-CD3, and a constant amount of anti-CD28 showed that low numbers of triggered TCRs required CD28 co-stimulation. Thus, IL-10 suppressed only those T cells that had low numbers of TCRs triggered and which required CD28 for proliferation.

IL-10 inhibits tyrosine phosphorylation of CD28 and phosphatidylinositol 3-kinase binding

Stimulation of CD28 by B7 surface molecules leads to tyrosine phosphorylation of CD28. Ligation of IL-10 receptor (IL-10R) at the time of CD28 stimulation inhibits tyrosine phosphorylation of CD28 as detected after 10 min^{33,57} (Fig. 2). The

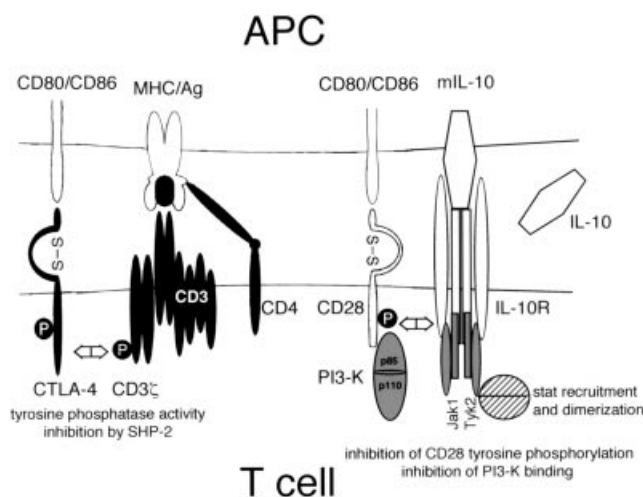


Figure 2. The molecular basis of T-cell suppression by IL-10. IL-10R inhibits CD28 tyrosine phosphorylation and phosphatidylinositol 3-kinase binding. IL-10 exerts its biological functions through the activation of Jak1 and Tyk2, the members of the receptor-associated Janus tyrosine kinases family and Stat1 and Stat3 and, in certain cells, Stat5. The effect of IL-10 on the inhibition of CD28 tyrosine phosphorylation is within minutes. This rapid activity does not require new protein synthesis. Similarly, CTLA-4, a negative regulator of T-cell function associates with the TCR complex ζ chain in T cells and utilizes the tyrosine phosphatase SHP-2 to dephosphorylate CD3 ζ chain.

inhibitory effect of IL-10 on CD28 appeared to be specific for the CD28 pathway, because IL-10 did not affect ZAP-70 tyrosine phosphorylation stimulated by CD3 cross-linking. As a consecutive event for signal transduction, phosphatidylinositol 3-kinase (PI3-K) should bind to CD28 by its p85 subunit. The association of CD28 with the PI3-K p85 molecule was inhibited by IL-10. This inhibition can be specifically blocked by preventing IL-10 binding to its receptor with an anti-IL-10 mAb.³³ PI3-K is a heterodimer that comprises an 85 000 MW regulatory and a 110 000 MW catalytic subunit possessing both protein serine-kinase and lipid-kinase activity.^{58,59} The p85 subunit contains a p110 binding site as well as two *src*-homology-2 (SH2) domains. Binding of PI3-K to CD28 occurs by direct interaction between SH2 domain motifs of p85 PI3-K and a (p)YXXM motif in the cytoplasmic part.⁶⁰ This requires CD28 tyrosine phosphorylation. From the primary protein sequence there is no indication that the CD28 cytoplasmic tail may display enzymatic activity.⁶¹ However, intervening protein tyrosine kinases, such as p56^{lck} and p59^{fyn} were demonstrated to phosphorylate CD28 at its 173Y position.⁶²

Previous studies demonstrated that IL-10 does not only inhibit T cells, it is also a potent inhibitor of activated monocytes and macrophages.^{45,46} Since monocytes and macrophages do not express CD28, the inhibitory impact of IL-10 is likely to occur through other mechanisms in non-T cells. IL-10 exerts its biological functions through the activation of Jak1 and Tyk2, the members of the receptor-associated Janus tyrosine kinases family and Stat1 and Stat3 and in certain cells Stat5.^{63,64} In monocytes, IL-10 was shown to induce expression of the suppressor of the cytokine-signalling 3 (SOCS3) gene that may play a role in inhibition of IFN-induced tyrosine phosphorylation of Stat1.⁶⁵ However, the effect of IL-10 on the

inhibition of CD28 tyrosine phosphorylation is already visible after 10 min. Obviously this does not require new protein synthesis and the mechanism of inhibition of CD28 tyrosine phosphorylation remains to be elucidated. Moreover, the identification of the second chain of the IL-10R, termed IL-10R2 or IL-10R β , as an essential molecule for the action of the IL-10R complex will further help to characterize IL-10 signalling.⁶⁶

IL-10R expression is controlled by CD28 and CD3 stimulation on T cells. T cells were stimulated with anti-CD28, anti-CD3 and IL-10. Surface expression of IL-10R α -chain, IL-10R β -chain and CD28 were determined by flow cytometric analysis. Freshly purified T cells express very few IL-10R. CD28 stimulation significantly enhanced IL-10R α and partially up-regulated IL-10R β expression after 24 hr.⁵⁷ By the same stimulation, CD28 itself was fully down-regulated by receptor internalization. Anti-CD3 stimulation strongly enhanced both IL-10R chains and CD28 expression. Surface CD28 expression and IL-10R α - and β -chain expression did not change by IL-10 stimulation in T cells. These results demonstrate a relationship between IL-10R and CD28 such that CD28 stimulation up-regulates the IL-10R expression rendering the cells more susceptible to IL-10-mediated suppression. Moreover, they demonstrate that the specific effect of IL-10 on inhibition of CD28 co-stimulation does not involve steric interactions of the two receptors and antibodies at the cell surface.

Resting human T cells contain very low or undetectable levels of IL-10Rs. These are increased after stimulation of T cells by anti-CD28, anti-CD3 and mitogens. Immunoprecipitation of CD28 and co-precipitation of the 110 000 MW IL-10R in lysates of activated T cells revealed an association of the two receptors.⁵⁷ In control experiments HuT 78 T cells, which do not express any of these molecules and Epstein-Barr virus-transformed B cells (BuB1), which only express the IL-10R, but not CD28, did not show association of IL-10R with CD28.⁵⁷ Recently, CTLA-4, a negative regulator of T-cell function was reported to associate with the TCR complex ζ chain in T cells.⁶⁷ The IL-10R/CD28 association, appears to be similar to CTLA-4/TCR ζ association. Both mechanisms have broad implications for the negative regulation of T-cell function and T-cell anergy in allergic as well as other inflammatory diseases (Fig. 2).

CONCLUSION

IL-10-induced peripheral T-cell tolerance during specific immunotherapy as well as natural antigen exposure represents a key event in the control of specific immune responses to high antigen doses. Both BV-SIT and PIT treatments decrease the antigen-specific IgE:IgG4 ratio in peripheral blood. The reactivation and modulation of distinct cytokine patterns in anergic T cells suggests a pivotal role of microenvironmental cytokines in the development of SIT. T-cell-secreted cytokines are essential for priming, survival and activity of inflammatory effector cells. Therefore, specific T-cell reactivity is directly involved in the pathogenesis of allergic inflammation. IL-10 not only induces anergy in T cells; it also inhibits the activation of inflammatory reactions by mast cells and eosinophils (Fig. 1).

The inhibition of the CD28 co-stimulatory pathway by IL-10 delineates a major mechanism in peripheral T-cell tolerance

(Fig. 2). IL-10 inhibits T-cell responses even at high antigen doses and the number of TCR triggered by the peptide-MHC complex on the surface of APC appears to be in the range of the requirement for co-stimulation. IL-10 efficiently increases the threshold for T-cell activation, rendering optimal conditions provided by professional APC and CD28/B7 interaction. In consequence, IL-10 induces an immunological unresponsive, anergic state in T cells.

Including various molecular mechanisms that generate T-cell tolerance, an increase in IL-10 production and suppression of CD28 co-stimulation-mediated T-cell proliferative and cytokine responses demonstrate important steps in tolerating exposure to high doses of exogenous antigen. These mechanisms have implications, which may reach beyond specific allergy treatment. Induction of specific anergy may also be of importance in autoimmunity and graft-versus-host disease. It can facilitate tumour growth, parasite survival and the development of acquired immune deficiency syndrome. The knowledge of this molecular basis is pivotal in understanding the equilibrated regulation of immune response and anergy to immunogenic agents and their possible therapeutic applications.

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