Beyond CTLA-4 and PD-1: Orphan nuclear receptor NR2F6 as T cell signaling switch and emerging target in cancer immunotherapy

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A B S T R A C T

Blockade of immune checkpoints has emerged as key strategy in the development of effective cancer therapies. In contrast to cell surface checkpoints like CTLA-4 and PD-1, however, additional cancer therapeutic targets are located inside the effector immune cells. Targeting these alternative checkpoints in cancer immunotherapy with the goal to strengthen the patient’s immune system are likely to extend the benefits of cancer immunotherapy in the near future. Along this line, we have defined and validated the orphan nuclear receptor NR2F6 (nuclear receptor subfamily 2 group F member 6, also called Ear-2) as an intracellular immune checkpoint in effector T cells. NR2F6 acts as a novel master switch of antitumor responses against both transplantable and spontaneous tumors in mice relevant for human cancer. NR2F6 directly represses transcription of key cytokine genes in T effector cells relevant for tumor cell rejection, such as IL-2, IFNγ and TNFα. Thus, in the presence of NR2F6, T cell activation is limited within the tumor microenvironment. This defines NR2F6 as a key checkpoint governing the amplitude of cancer immune surveillance. Based on our study, an approach shall be initiated to identify low molecular weight compounds that selectively interfere with NR2F6 function in the clinic.

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1. Introduction

Cancer is a severe health problem with increasing prevalence in first world countries. Cancer comprises multi-factorial disorders, and due to the complexity of the disease, a mechanistic understanding of the numerous acquired hallmarks of cancer is limited. However, malfunction of cancer immune surveillance is emerging as a crucial event in cancer etiology. Historically, in the late nineteenth century, William B. Coley made a pioneering observation of tumor shrinkage and disappearance following injection of bacterial products in and around tumors. Paul Ehrlich suggested the crosstalk between the immune system and cancer in 1909; a half century later, Burnet and Thomas formulated the cancer immune surveillance hypothesis [1].

Evidence, however, this hypothesis had to be abandoned shortly thereafter. Today’s refined technologies including mouse models of cancer immunity have validated the key importance of immunity in cancer progression. It is well established that mice who lack essential components of the innate or adaptive immune system (e.g., Rag1-deficient mice) are significantly more susceptible to the development of spontaneous or chemically induced tumors. Consistently, the immune system of transplant patients who receive immunosuppressive drugs is less able to detect and destroy cancer cells [2].

The immune system has been demonstrated to serve key roles in the prevention of tumors. It can protect the host from virus-induced tumors by eliminating or suppressing viral infections. The timely elimination of pathogens and prompt resolution of inflammation can prevent the establishment of an inflammatory environment known to promote tumorigenesis. Finally, the immune system can specifically identify and eliminate tumor cells on the basis of tumor-specific antigens or molecules induced by cellular stress, a phenomenon called tumor immune surveillance [3]. However, immune surveillance represents only one dimension of the complex interplay between the immune system and cancer. New results demonstrate that the immune system also promotes the emergence of tumors with reduced immunogenicity capable of escaping immune recognition and destruction. These
two faces of the immune system during tumorigenesis prompted a refinement of the cancer immune surveillance hypothesis into one termed “cancer immunoeediting,” encompassing both the potential host-protective and the tumor-sculpting functions of the immune system throughout cancer development [4].

2. T cell immunology meets oncology

CD8+ cytotoxic T cells recognize and kill tumor cells in an MHC-restricted and perforin-dependent manner. Mechanistically, tumor cells and/or tumor antigens are taken up, processed, and cross-presented to T cells by professional APCs and induce CD8+ T cell responses [2,5]. Nevertheless, stimulation via the tumor antigen-specific TCR alone is apparently insufficient for activation of naïve as well as memory CD8+ T cells. Therefore, a co-stimulatory signal (CD28 on the T cell—CD80/CD86 on the APC) is considered essential for a full activation scenario. This leads to proliferation and differentiation of naïve CD8+ T cells into effector cytotoxic CD8+ T cells (CTL) and memory CD8+ T cells, resulting in production of cytokines such as IFNγ and the effector molecules perforin and granzyme B, mediating direct cytolysis activity on the targeted tumor cells [3]. Several groups have shown the critical importance of CD4+ T cells in the induction and maintenance of antigen-specific memory CD8+ T cells [6-8]. In the absence of T helper cells, memory CD8+ T cells exhibit impaired functionality, persistence, and most importantly, the ability to efficiently control a tumor challenge. Especially TH1 cells that secrete high levels of IFNγ, TNFα, and IL-2 cooperate with the cell-killing functions of CD8+ T cells, and are able to induce up-regulation of MHCⅠ and MHCⅡ on APCs, thereby strongly enhancing the duration and magnitude of CTL responses. In detail, the release of inflammatory cytokines such as IL-2 helps to expand the population of tumor antigen-specific CTLs [9]. Additionally, TH1 cells directly contribute to antitumor immunity via the release of IFNγ, whereas TNFα induces cytotoxic effects independent of B, NK or CD8+ T cells [10,11]. Additionally, activated CD4+ T cells recognize tumor-infiltrating macrophages in an MHCⅡ-dependent manner, converting IL-10 (interleukin-10)-producing M2 macrophages into IFNγ-producing M1 macrophages [2]. On the other hand, regulatory T cells (CD4+CD25+Foxp3+) that normally prevent autoimmunity by maintaining self-tolerance contribute to immune suppression by dampening the antitumor immunity caused by CD4+, CD8+ T cells, DC (dendritic cells) and NK (natural killer) cells at the tumor site. Inhibitory cytokines (TGFβ, IL-10, and IL-35), cytosis of effector T cells via granzyme-B and perforin, and IL-2 deprivation lead to Treg-mediated immunosuppression [9]. Moreover, other cells of the adaptive (TH2, TH17, NKT cells) as well as innate immune system (DC, NK cells, myeloid derived suppressor cells, and tumor-associated macrophages) critically contribute to tumor immune surveillance.

Consistently, the presence and number of tumor infiltrating lymphocytes (TILs) is a favorable prognostic marker in numerous cancers. This applies in particular to melanoma, ovarian carcinoma and colon carcinoma [12]. The presence of Treg cells in the tumors of patients with ovarian carcinoma or melanoma, on the other hand, is a predictor of reduced survival [13,14]. However, the exact molecular mechanisms by which tumors mediate immunosuppression remain under intense investigation.

3. Immunotherapy in cancer

Anticancer treatment, next to basic surgery, is generally categorized into five different classes: radiation therapy, where deposited energy can kill remaining cancer cells or cause genetic changes resulting in cancer cell death [15]; chemotherapy, which involves diverse groups of cytotoxic drugs that interfere with cell division and DNA synthesis [16]; hormonal therapy, where drugs interfere with growth survival signaling through hormone receptors on cancer cells [17]; targeted therapy, which consists of a novel group of antibodies or small-molecule kinase inhibitors that specifically target growth signaling pathways in defined cancer; and immunotherapy, which targets the induction and/or augmentation of antitumor immune responses [12,18].

Over the past decades, many observations like spontaneous remissions, higher cancer incidence in immunosuppressed patients, tumor-specific antigens and lymphocytes, gave a strong push to research in developing efficacious immunotherapy, and resulted in successful breakthroughs in drug development and treatment approval against cancer [19]. The advantage of cancer immunotherapy is the exquisite power and specificity of the immune system in the treatment of malignancy, especially via the cytotoxic T lymphocyte response. However, because tumors frequently interfere with effective antitumor immune responses, immunotherapy has to develop strategies to overcome this hurdle [18]. Insights into tumor-specific immune responses were obtained in patients with melanoma, since the majority of tumor immunology studies have been performed on this tumor type. Although clinical remissions have consistently been observed using immunotherapies, overall the efficacy remained below a threshold that justified their use in the general patient population [19,20]. Nevertheless, there are a few quite distinct strategies that help boost the immune system against cancer: vaccines, cytokines, antibodies, immune checkpoint blockade inhibitors, adoptive cell transfers, and chimeric antigen receptor-modified T-cell therapy [12,21].

Few vaccines exist that provide protection from cancer. Either prophylactic vaccines, such as the quadrivalent HPV vaccine preventing human papilloma virus infection in young girls and boys [22], or therapeutic vaccines based on antigen-presenting cells, like dendritic cells, leading to a specific immune response against introduced tumor antigens, like sipuleucel-T [23]. The success of sipuleucel-T has encouraged the development of other therapeutic prostate cancer vaccines, including the tumor-cell based GVAX-Pca and the viral vaccine Prostvac [24]. Many vaccine approaches, including tumor cell vaccines, peptide vaccines, DNA vaccines and dendritic cell vaccines can induce immune responses, but they have been basically unsuccessful in achieving any clinical responses [19]. Over the years it has become clear that cancer cells have different ways of actively suppressing the immune system at the tumor site, which in fact makes effective vaccinations almost impossible [20].

Cytokines, on the other hand, directly modulate the immune response and can be used as biological drugs. Two commonly approved cytokine therapies are IFNα against leukemia, sarcoma, lymphoma, CML and melanoma [25], and IL-2 against melanoma and renal cell carcinoma [26]. Cytokine treatment, however, is so far not a common treatment, except in some adjuvant settings, due to its high toxicity in patients. The underlying toxicity of IL-2 results from a capillary leak that leads to fluid extravasation into visceral organs that can compromise their function, as well as fever, chills, malaise, and arthralgia [27]. The most common toxicity of systemic administration of type I interferons, namely IFNα are fatigue, anorexia, hepatotoxicity, flu-like symptoms and severe depression [28].

“Passive” immunotherapy with monoclonal antibodies (mAbs), raised against specific antigens, present on tumor cell surfaces, or directly stimulating the immune response, has outpaced “active” immunotherapy with antitumor vaccines. Many therapeutic antibodies blocking for example CD20 (ofatumumab, rituximab), EGFR (trastuzumab, panitumumab, nimotuzumab, cetuximab), and VEGF (bevacizumab) [20,29] are approved by the FDA. The use of mAbs as “biologics” for therapy of cancer became a great success story of the past decade.
In contrast to the above-mentioned mAbs targeting mainly tumor cells, immune checkpoint therapies, which target regulatory pathways in immune cells in order to enhance antitumor immune responses, have led to even more remarkable clinical advances and provided an entirely new handle in the “war against cancer” [30]. The immune system has several checkpoints as a safeguard to keep itself from attacking normal cells in the body. Cancer cells apparently take advantage and hitchhike these immune checkpoints to avoid being attacked by the immune system [20]. Three immune checkpoint-blocking agents have now been approved by the FDA for the treatment of melanoma in the clinic—one antibody against cytotoxic T-lymphocyte-associated protein 4 (CTLA-4; ipilimumab) in 2011, and two antibodies against programmed cell death protein 1 (PD-1; pembrolizumab and nivolumab) in 2014 [31]. But other antibodies, for example those blocking the ligand of programmed cell death protein 1, PD-L1, or targeting other immune checkpoints like surface receptor Tim3, might prove to be successful in the near future [32,33].

In detail CTLA-4 is upregulated upon T cell activation, and like CD28, binds B7 molecules with higher affinity, thus leading to down-regulated T cell activation responses via an ectodomain competition [34]. Thus, blocking its interaction with B7 molecules might allow T cell responses to persist sufficiently to achieve tumor eradication [30]. Unfortunately, however, CTLA-4 blockade has been associated with dramatic autoimmune toxicities as on-target side effects, such as colitis, dermatitis, hypophysitis, or pulmonary alveolitis [12]. PD-1 is also an inhibitory surface receptor upregulated on activated T cells, as well as on B cells and monocytes. PD-1 has two ligands, PD-L1 (expressed in immune and nonimmune tissue) and PD-L2 (expressed on APCs). PD-L1 can be upregulated by both IFNα/β and IFNγ, leading to a high expression in a variety of inflamed tissues, tumors as well as TILs [35]. PD-L1 exerts its function by binding to PD-1 and B7.1 (CD80), both of which are negative regulators of T cell activation signaling, thereby suppressing T cell migration, proliferation and secretion of cytotoxic mediators [32,36]. Treatment with the humanized antibody MDPL3280A in a small percentage of patients with high levels of PD-L1 appears most effective due to the re-invigoration of pre-existing immunity by tumor antigen-specific TILs [32]. Finally, combination therapies may overcome the limitations of anti-CTLA-4 and anti-PD-1/PD-L1 monotherapies: In fact, anti-CTLA-4 apparently drives T cells towards the tumor site, resulting in increased numbers of T cells and an increase in IFNγ levels. This, in turn, induces expression of PD-L1 in the tumor microenvironment, subsequently inhibiting antitumor T cell responses. However, it also increases the chance of clinical benefit from anti-PD-1 or anti-PD-L1 therapy regimens in these patients. Combination therapies thus in deed appear to induce a superior immunogenic tumor microenvironment with higher clinical benefit [37].

Adoptive T cell therapy (ACT) relies on the in vitro expansion of endogenous, cancer reactive T cells (mainly CD8+ cytotoxic T cells), which are harvested from cancer patients, manipulated, and then reintroduced as a mechanism for generating productive tumor immunity [12]. ACT using ex vivo expanded T cells can induce tumor regression in patients with advanced melanoma [38]. T cells transduced with tumor antigen-specific T cell receptor transgenes have been used to treat patients with melanoma [39] or B cell lymphoma [40], thereby bypassing the need to expand tumor-specific T cells ex vivo. Nevertheless, the therapeutic efficacy of ACT appears to be limited by immunosuppressive mechanisms within the tumor-bearing host. While tumor-specific immune responses are frequently observed, sustained clinical benefits are documented in only a small fraction of patients [12]. In line with ACTs, a new type of immunotherapy, known as chimeric antigen receptor (CAR)-modified T cell therapy, has recently emerged. T cells are removed from the patient’s blood and genetically altered to have specific antigen receptors that are specific to tumor antigens on the surface of cancer cells. Such genetically modified T cells are expanded and infused back into the patient’s blood, where they induce a targeted immune attack against the cancer cells. CAR T cell therapy for ALL (Acute lymphoblastic leukemia) combines chemotherapy with ex vivo reprogramming of previously removed T cells, genetically transduced with a CD19 antigen-specific CAR. The therapy is the first effective treatment for relapsed ALL patients in adults and children, where up to 88% of the patients achieved remission [21,41].

4. Beyond current cancer immune therapy regimens

Nuclear receptors (NRs) are transcription factors that have been shown to be essential for both pro- and anti-inflammatory T cell responses. NRs directly bind DNA and regulate gene expression in response to ligands. NRs can transduce signals from glucocorticoids, mineralocorticoids, sex steroids (estrogen, progesterone, and androgen), thyroid hormones, or vitamins either as homo- or heterodimer. All of the NRs have common structural features, which include a central DNA-binding domain (DBD) responsible for targeting the receptor to highly specific DNA sequences, a ligand-binding domain (LBD), which recognizes specific ligands, a functional transactivation site (AF-1) for gene regulation, a ligand inducible activation function (AF-2) for co-activator and co-repressor interactions, and a hinge region, connecting the DBD with the LBD influencing intracellular trafficking and subcellular distribution [42,43]. Increasing evidence shows that perturbations of NR signaling pathways engaged downstream of the TCR underlie the development of several immune pathologies including cancer.

We discovered the orphan nuclear receptor subfamily 2, group F, member 6 (NR2F6) to act as a direct signaling intermediate and threshold regulator of TCR/CD28-mediated signal transduction in primary mouse and human CD3+ T cells. NR2F6 is termed orphan nuclear receptor because endogenous ligands are undefined [44]. NR2F6 thereby functions as a critical protein kinase C (PKC) effector substrate and essential non-redundant negative regulator of adaptive immunity [45,46]. NR2F6 represents a widely expressed transcription factor and is a member of the COUP-TF (chicken ovalbumin upstream promoter transcription factor) family. This COUP-TF family of NRs has two other mammalian family members, NR2F1 (COUP-TFI) and NR2F2 (COUP-TFIi). COUP-TF family members as homo- or hetero-dimers are pre-bound to cognate DNA response elements, i.e. AGGCCA direct or inverted repeats with various spacing and are thought to act as a repressor for locking out stochastic transcriptional activation of pro-inflammatory cytokine genes. In transfected cell line studies, the role of NR2F6 was first described in 2003 by Liu et al., referring to an inhibitory effect of EAR2/NR2F6 on renin transcription [47,48]. Of note, NR2F6 was reported to be overexpressed in colorectal cancer biopsies [49]. Warnecke et al. described the first Nr2f6 knockout mouse in the context of brain development in 2005. Mice were born alive and fertile but lacked <70% of the locus coeruleus leading to a mildly altered circadian behavior demonstrated by delayed entrainment to shifted light-dark cycles, less adaption to daytime feeding schedules, and increased pain sensitivity [50]. Of note, Ichim et al. describe a candidate regulatory role of NR2F6 during T cell development. Bone marrow reconstitution experiments with forced overexpression of recombinant NR2F6 resulted in limited T cell development and decrease in thymus size and cellularity [51]. Our investigations with Nr2f6 knockout and transgenic overexpression in mice, however, excluded any defect in thymus development (unpublished data). Importantly, however, the NR2F6-dependent transrepression pathway(s) of pro-inflammatory cytokines in particular appear(s) to play a key role in local and systemic inflammation.
5. **Biological relevance of NR2F6 in the regulation of T cell-mediated immune responses**

NR2F6 has a critical regulatory function in the adaptive immune system [45], as it negatively controls T cell receptor/CD28–mediated transactivation of key transcription factors such as nuclear factor of activated T cells (NFAT) and the activating protein 1 (AP-1). We observed that during TCR activation, a PKC/NR2F6 module and a regulatory phosphorylation on the NR2F6 DBD function as a critical feedback mechanism [45]. Upon high affinity antigen receptor signaling, PKC-mediated phosphorylation of Ser-83 within the DBD of NR2F6 abrogates the DNA-binding capacity of NR2F6, thereby promoting unopposed DNA binding of NFAT/AP-1 transcription factors at the critical cytokine gene loci [45,46]. Our most recent research confirms the T cell–intrinsic “NR2F6 restraint mechanism” to be in control of the initiation, magnitude and duration of IL-2, IFNγ and TNFα cytokine gene expression of effector T cells in vitro and in vivo [52]. Collectively, these findings support the concept that the integrity of a PKC/NR2F6 signaling complex is mandatory for proper T cell effector functions and fine-tuning of an adaptive immune contexture favoring continuous tumor cell elimination.

Mechanistically, NR2F6 acts as transcriptional repressor in TH0 and TH17 cells via direct binding to the Il2 and Il17a promoter loci, thus suppressing DNA accessibility of NFAT and AP-1 at this promoter site. NR2F6 facilitates repression of cytokine production also in TH1 CD4+ as well as CD8+ effector T cells, thereby affecting T cell activation and effector outcomes. In the experimental autoimmune encephalomyelitis (EAE) model, Nr2f6-deficient mice displayed significantly augmented disease progression [45], validating in vivo an involvement of NR2F6 in induction and/or maintenance of autoimmunity. The exact molecular pathway by which NR2F6 impairs the transcriptional amplitude of NFAT/AP-1 gene transactivation including all target genes of NR2F6 is currently under investigation.

Longstanding evidence, however, suggests that activated NFAT proteins must exceed a certain threshold level before they can initiate transcription [53]. Because NFAT proteins are master regulators of T cell–derived cytokine transcription, it makes sense for NFAT to function within a negative feedback loop that is capable of down-modulating immune responses by inducing and/or maintaining an anergic state. Importantly, NFAT2 is expressed at only low levels in resting T cells, but is markedly induced upon T cell activation, to augment and sustain NFAT–regulated gene transcription, which is important for the development and function of effector T cells. The induction of NFAT2 upon T cell activation is mediated primarily by constitutively expressed NFAT1 [54,55]. Along these lines, transcription factor (TFs) proteins are well established to directly
compete with other TFs to regulate promoter transcription by shifting the stoichiometric balance of promoter occupancy. Thus, in this model, NR2F6 expression levels may significantly affect the threshold level of DNA-bound NFAT proteins on key promoter loci that are required to increase NFAT-driven gene transcription and prevent peripheral tolerance. Furthermore, NR2F6 thereby could directly antagonize NFAT1- and NFAT2- modulated transcription [55]. By simultaneously inhibiting Nfat2 transcription directly via its NFAT1-mediated promoter activation in a feed-forward loop, NR2F6 might be able to govern cytokine expression. In this model of T cell physiology, based on its ability to inhibit NFAT1-mediated transcription, NR2F6 would be able to intervene at two levels. First, NR2F6 modulates initial NFAT1-mediated immune responses by binding to cytokine promoters directly antagonizing NFAT1, where it is thought to actively suppress transcription [45,46]. Second, and as a direct consequence, the second wave of NFAT activation might be suppressed by inhibiting the upregulation of NFAT2 in response to T cell activation, thereby maintaining the level of activated NFAT proteins below that required for transcriptional activation. The abrogation of NFAT1 binding in the presence of NR2F6 would serve as a feed-forward mechanism that could suppress the activation-induced expression of Nfat2 mRNA and protein in T cells. Similarly to the If2 and I117a promoters, candidate NFAT/NR2F6 combi-sites, as defined by computer analysis in silico, where NR2F6 was found to bind to DNA immediately adjacent to NFAT, are located in the Nfat2 promoter locus. NR2F6 may therefore augment its effect on NFAT-driven promoters by limiting the amount of de novo expressed NFAT2 that is available, thereby maintaining its levels below the threshold required for transcriptional activation, which is especially important in T cell effector function [46]. Of note, this is also expected to affect tolerance induction during suboptimal TCR stimulation conditions. Indeed, primary T cells that are derived from Nr2f6-deficient T cells did not respond to induction of classical energy by the Ca2+ ionophore ionomycin and are significantly less sensitive to activation-induced cell death (AICD) that is induced by CD3 stimulation in vitro ([45] and unpublished results).

Of note, NR2F6 is strongly upregulated in chronically activated T cells as an "exhaustion factor", further strengthening its important negative regulatory function in adaptive immunity. Nothing, however, was known about a negative regulatory role of NR2F6 in other T cell-mediated immune diseases such as cancer. We have now identified NR2F6 to be pre-bound to its hormone response elements (HRE) on defined gene loci in steady state. Investigating NR2F6 in CD3+ T cell biology helped define its role in tumor immunity in various mouse cancer models. Analysis of the molecular mechanism underlying NR2F6 function revealed a T effector cell intrinsic role of NR2F6, repressing tumor rejection by reducing tumor T cell infiltration as well as cytokine production at the tumor site [52]. Employing Nr2f6-deficient mice and T cells to investigate the effect of NR2F6 on the growth of endogenous and transplanted tumors, we were able to demonstrate that the absence of NR2F6 in T cells leads to a drastic increase in the infiltration of various tumors by immune cells, specifically T cells, showing an overactivated phenotype with increased secretion of IL-2, IFNγ and TNFα as key effector cytokines by both TH1 CD4+ and CD8+ effector T cells. As a consequence of increased immune cell infiltration and activation, Nr2f6-deficient tumor-bearing mice show significantly reduced tumor growth due to their hyperactive cancer immunity. Thus, this study identifies NR2F6 as a critical negative regulator of T cell immune responses and inhibition of NR2F6 as a potential cancer therapeutic target in the clinic for enhancing antitumor immune responses (Fig. 1).

Albeit nuclear receptors directly regulate gene expression in response to lipophilic ligands, nearly half of all human NRs including NR2F6 lack valid information on endogenous ligands. Because mutations designed to reduce the size of the evolutionarily conserved LBD pocket or to disrupt co-repressor interactions has been shown to significantly reduced NR2F6 transcription activity [46], the LBD of NR2F6 appears essential for its transcriptional repressor activity. This biochemical evidence strongly suggests that endogenous NR2F6 ligands, although currently undefined, may exist and presumably modulate the active conformation to induce homo- and/or hetero-dimerization and/or recruitment of co-activators/co-repressors. As an approach towards this goal, affinity chromatography of cellular lipidomes with the NR2F6 LBD and subsequent functional assays are currently performed in order to define candidate ligands of NR2F6. If successful, its endogenous ligands will help to resolve the regulation mechanisms for the LBD of NR2F6. Furthermore, a defined NR2F6-ligand interaction would provide valuable proof-of-principle of NR2F6 drugability that will aid in the development of a "small molecule checkpoint blockade drug" for immunomodulation in the clinic.

In conclusion and because there is a high scientific interest to explore novel cancer immune-therapeutic avenues with the ultimate goal to strengthen the patient’s immune system for a longer progression-free survival, our validation of a both alternative and potentially druggable immune checkpoint may extend the benefits of clinical treatment in the future.

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