Journal of Pathology
J Pathol 2013; **229:** 168–175
Published online in Wiley Online Library
(wileyonlinelibrary.com) DOI: 10.1002/path.4123

INVITED REVIEW

Regulation of the immune and inflammatory responses by the 'atypical' chemokine receptor D6

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Abstract

Chemokines and their receptors are key regulators of leukocyte migration and intra-tissue accumulation under both homeostatic and inflammatory conditions. Regulation of chemokine-dependent responses, particularly those relating to inflammation, is essential to avoid the development of inflammatory and autoimmune pathologies. Recently, a new subfamily of chemokine receptors referred to as the 'atypical' chemokine receptors has emerged, members of which have been shown to play important roles in controlling *in vivo* chemokine biology. Here we review the basic biology of the chemokine and chemokine receptor family, introduce the topic of 'atypical' chemokine receptor biology and focus specifically on the best-characterized of the 'atypical' chemokine receptors, D6. D6 is a 'scavenging' receptor for inflammatory CC chemokines and plays a central role in the resolution of *in vivo* inflammatory responses. We describe the biology, biochemistry and pathological relevance of D6 and outline emerging data suggesting that it has additional important roles in integrating innate and adaptive immune responses.

Keywords: chemokine; inflammation; immunity; decoy receptor

Received 6 September 2012; Revised 3 October 2012; Accepted 3 October 2012

No conflicts of interest were declared.

Introduction

In vivo migration of leukocytes is a carefully orchestrated process, mainly regulated by proteins belonging to the chemokine family [1,2]. This family is defined on the basis of a conserved cysteine motif and is divided into four subfamilies, CC, CXC, XC and CX3C, on the basis of the specific nature of the cysteine motif. Chemokines are a vertebrate 'invention' and their primordial role appears to have been to regulate stem cell migration and tissue compartmentalization during embryogenesis [3,4]. The system has now evolved to the point where most mammals have almost 50 chemokines and 18 chemokine receptors [5], involved, sometimes in extremely subtle ways, in regulating *in vivo* leukocyte migration. Accordingly, the biology of chemokines and their receptors is complex, but can be somewhat simplified by defining them as being either homeostatic or inflammatory, according to the *in vivo* situations in which they function [2,6]. Thus, homeostatic chemokines and their receptors regulate basal leukocyte trafficking to peripheral tissues and secondary lymphoid organs and are generally regarded as being expressed at steady levels. Homeostatic chemokine/receptor function *in vivo* is perhaps best exemplified by considering the chemokines CCL19 and 21 and their receptor CCR7 [7]. These ligands are expressed within lymph nodes and on lymphatic endothelial cells and their receptor is expressed on cells destined for lymph node homing. This combination of ligand and receptor therefore directs cells to secondary lymphoid organs from peripheral tissues and is essential for the establishment of such organs during the development and migration of antigen-presenting cells to lymph nodes during adaptive immune responses. Other homeostatic chemokine receptors include CCR9, CCR10 and CXCR5, which are important, individually, as parts of cellular address codes [8] for specifying the tissue-specific homing of individual leukocyte subtypes. Thus, CCR9 supports the migration of leukocytes to the gut [9], CCR10 to the skin [10] and CXCR5 supports the migration of B cells to lymph node follicles [11]. Importantly, there is very little redundancy built into the homeostatic chemokine and chemokine receptors, and mice deficient in these receptors, or ligands, invariably display important basal phenotypes. In contrast, inflammatory chemokines and their receptors are not normally expressed at significant levels but are induced to high

levels following tissue infection, insult or injury. They then support the recruitment of inflammatory leukocytes to inflamed or damaged sites and are eventually transcriptionally silenced as a prelude to inflammation resolution. Typical inflammatory chemokines include CCL2, CCL3 and CCL5 as well as CXCL1, CXCL2 and CXCL8. In contrast to the homeostatic chemokine receptors, there is considerable apparent 'redundancy' built into the inflammatory chemokine/receptor family [12,13]. Accordingly, mice with deletions in individual inflammatory chemokines and their receptors tend to display partial, and overlapping, phenotypes. This issue has hampered our abilities to understand the overall orchestration of a chemokine-driven inflammatory response, and has also contributed to the current problems associated with rationally targeting the inflammatory chemokine receptor system in inflammatory pathologies using receptor-specific therapeutics.

'Atypical' chemokine receptors

Whilst the classical signalling chemokine receptors described above have been known for some time [5], more recently a novel subfamily of chemokine receptors, entitled the 'atypical' chemokine receptor family, has emerged, members of which appear to be essential contributors to the regulation of chemokine-dependent responses [14,15]. The 'atypical' chemokine receptor family (Table 1) is defined on the basis of the apparent inability of its members to signal in response to ligand binding in the same way as the typical signalling chemokine receptors [5]. It is worth mentioning that classical signalling chemokine receptors and atypical chemokine receptors group to a cluster phylogenetically within the type A G protein-coupled receptors [16]. As apparent from their ligand binding profiles, CCX-CKR is likely to play an as-yet poorly defined role in the orchestration of homeostatic chemokine function [17–19], while CXCR7 has clear developmental roles [20–22] related to the key and ancient role for one of its ligands, CXCL12, in stem cell migration and tissue compartmentalization during embryogenesis [23]. Data on CCRL2 binding profile and function are still controversial, but recent evidence of binding properties for CCL19 would suggest its involvement in the regulation of the CCR7 axis [24]. The other two receptors, DARC and D6, are characterized by promiscuous binding of inflammatory chemokines. Indeed, DARC is the only vertebrate chemokine receptor known to bind chemokines belonging to more than one subfamily [25]. Current *in vivo* data suggest complex roles for DARC in regulating chemokine presentation and bioavailability [26–28], but as yet we have no coordinated understanding of its role in inflammation. In contrast, D6 plays well-characterized roles in the regulation of the *in vivo* inflammatory response in ways that are now extensively understood. D6 therefore represents the best-characterized member of the 'atypical' chemokine receptor family and will thus be focus of the remainder of this review. Readers are referred to other reviews for further insights into the biology and biochemistry of the other members of the 'atypical' chemokine receptor family [14,15].

Table 1. Ligands for the 'atypical' chemokine receptors

CCX CKR	**CCL19, 21, 25**
CXCR7	CXCL11, 12
DARC	CCL1, 2, 5, 7, 8, 11, 13, 14, 16, 17, 18; CXCL5, 6, 8, 9, 10, 11, 13
D6	CCL2, 3, 4, 5, 7, 8, 11, 12, 13, 14, 17, 22, 23, 24
CCRL2	CCL19

The identification of D6

D6 was simultaneous identified by us [29,30] and one other group [31], using degenerate genomic- and cDNA-based cloning strategies, respectively. The closest D6 homologues are other chemokine receptors and, indeed, the *D6* gene sits within the major chromosomal locus incorporating many of the other chemokine receptors in the mouse (chromosome 9) and human (chromosome 3) [3], indicating that D6 has evolved from within the chemokine receptor family. The cloned cDNA encodes a typical seven-transmembrane-spanning (7TM) receptor, with distinctive alterations in conserved motifs that result in its inability to signal in response to ligand in a manner equivalent to that seen with the classical chemokine receptors. The issue of D6 signalling will be dealt with in more detail below.

D6 binds essentially all inflammatory CC chemokines but not homeostatic CC chemokines or chemokines belonging to any of the XC, CXC or CX3C subfamilies [29,30,32–34]. Interestingly, all D6 ligands are characterized by the presence of a proline residue in the second amino acid position (P2) [34,35]. Indeed, there exists a non-allelic variant of the prototypic inflammatory CC chemokine, CCL3, in the primate genome, which lacks this proline and which therefore binds, with very poor affinity, to D6 [35,36]. The importance of this evolved variant in the context of an inflammatory response is not yet apparent. Intriguingly, the requirement for a proline in the P2 position highlights D6 ligands as being potential substrates for the enzyme CD26 [37]. Accordingly, CD26 activity is likely to neutralize the ability of inflammatory CC chemokines to bind to D6 and also alter their affinity of binding to the signalling chemokine receptors in complex ways. This is likely to have important, but currently poorly understood, effects on the orchestration of an on-going inflammatory response.

D6 expression patterns

Initial northern blotting and PCR-based analyses revealed expression of D6 in the skin, gut, lung and placenta [29,30]. Therefore, in terms of expression, D6

positions itself in barrier tissues, and the importance of this will be described below. Further analysis using immunohistochemistry has demonstrated that the primary site of D6 expression in resting tissues is lymphatic endothelium [38]. Importantly, not all lymphatic vessels express D6, suggesting that this gene is regulated on lymphatic endothelium and, whilst the precise nature of this regulation has yet to be defined, our preliminary analyses indicate that D6 expression on lymphatic endothelial cells is up-regulated by a variety of pro-inflammatory mediators, including IL-6 and IFNγ (unpublished data). It is likely, therefore, that D6 is up-regulated on lymphatic endothelium specifically in the context of on-going inflammatory responses. In addition to lymphatic endothelial cells, in the placenta the major site of D6 expression is the syncytiotrophoblast [39,40]. In addition, D6 is expressed in a range of leukocyte subtypes, most notably subsets of dendritic cells and innate-like B cells [41,42]. Leukocyte expression is regulated by both inhibitors and stimulators of inflammatory responses, with TGFβ increasing expression and inflammatory molecules such as LPS reducing expression [42]. We have also recently described D6 expression on keratinocytes from psoriatic epidermis [43], suggesting that expression may be more widespread, and context-dependent, than we currently understand.

D6 structure–function properties

As mentioned, D6 is a promiscuous chemokine receptor able to bind, with high affinity, a large panel of inflammatory CC chemokines, including most ligands for the classical chemokine receptors CCR1–CCR5 (Table 1). Although the precise binding sites have not been mapped, the D6 N-terminal domain contains several acidic amino acids and sulphated moieties likely involved in ligand recognition [44–46]. The N-terminal domain is also glycosylated (N-linked), although this post-translational modification appears not to influence receptor expression and ligand binding [44]. Several structural elements conserved in classical chemokine receptors show significant modifications in D6. The well-conserved DRYLAIV motif, located at the boundary of the second intracellular loop and transmembrane region 3 (TM3) in the signalling chemokine receptors, is modified to DKYLEIV in all mammalian D6 sequences [14]. Importantly, substitution of the negatively-charged glutamic acid in D6 with alanine resulted in partial recovery of ligand-induced calcium signalling, suggesting that altered DRY motif may be partially responsible for the lack of conventional signalling activities of D6 [47]. Similarly, whilst the T–x–P motif conserved in TM2 of classical chemokine receptors and involved in receptor activation is also conserved in D6, the flanking aspartic acid residue, conserved in TM2 of most 7TM and required for activation, is substituted by an asparagine residue in D6. Although their relevance for D6 signalling properties has not been investigated in detail, these alterations are seen in all mammalian D6 sequences currently available, suggesting that these are not loss-of-function mutations and likely play specific roles in D6 biology.

The chemokine-scavenging properties of D6 are intimately linked to its trafficking. In resting conditions, D6 is predominantly located in intracellular perinuclear compartments [44,48], is constitutively internalized in Rab5-positive vesicles through clathrin-coated pits by a dynamin-dependent mechanism, and is then targeted to early endosomes [48,49]. Internalized D6 molecules are recycled back to the plasma membrane, partially via a Rab4-dependent rapid recycling pathway and partially via a slow Rab11-dependent recycling pathway [49]. In contrast to the classical chemokine receptors, ligand engagement increases D6 expression on the cell surface due to the mobilization of the intracellular pool via the Rab11-dependent pathway [49]. Constitutive cycling and ligand-dependent up-regulation are mechanisms allowing rapid modulation of ligand uptake and degradation [50]. In classical chemokine receptors, internalization requires β-arrestin recruitment to the phosphorylated C-terminal domain [51,52]. As compared to classical chemokine receptors, D6 is characterized by a longer C-terminal domain with a serine cluster, involved in receptor trafficking and ligand degradation, whose deletion or mutation to alanine strongly reduces D6 stability by its targeting to lysosomal compartment, presumably through ubiquitination of two conserved lysine residues [14,53]. D6 scavenging function is also associated with β-arrestin relocalization on the cell surface, although discrepant results have been obtained on the role of receptor phosphorylation in this event [53,54]. For a long time β-arrestins have been considered only as a mechanism to switch of G protein signalling and desensitize receptors [55]. In contrast, recent data have clearly established their role as adaptor proteins involved in signalling, and 7TM receptors are now thought to signal through G protein- and β-arrestin-mediated pathways in a balanced fashion [56]. Interestingly, modification of the DRY motif of chemokine receptors has been shown to interfere with their G protein signalling activity, resulting in association with β-arrestins [57]. As mentioned, D6 presents similar modification in the DRY motif and associates with β-arrestins, leading to the hypothesis that it may operate as a β-arrestin-biased receptor. Indeed, we have reported evidence of non-conventional signalling activities for this receptor. In particular, we have shown that D6 trafficking properties are affected by D6 ligands that are driven to degradation after receptor engagement, while other chemokines, including protease-inactivated chemokines, bind with similar affinity to the receptor but have no influence on its cellular distribution and are not degraded [34,49]. Recently, we have collected evidence that D6 adaptive up-regulation and chemokine scavenging activity result from its ability to activate a β-arrestin2-dependent Rac1–PAK1–LIMK1

signalling pathway, leading to cofilin phosphorylation and cytoskeletal reorganization (unpublished results).

D6 and the regulation of the *in vivo* inflammatory response

The ability of D6 to scavenge, internalize and degrade inflammatory chemokines with high efficiency highlights D6 as a potential regulator of the resolution of *in vivo* inflammatory responses. Accordingly, D6-deficient mice were generated to test this hypothesis. D6-deficient mice have no overt resting phenotype but display a marked inability to resolve inflammatory responses in all tissues in which D6 is normally expressed.

- *The skin*. Initial studies demonstrated that after either topical application of the phorbol ester TPA [58], or induction of granuloma development by subcutaneous injection of complete Freund's adjuvant (CFA) [59], D6-deficient mice demonstrate an impaired ability to resolve cutaneous inflammatory responses compare to wild-type (WT) mice. In the context of the TPA application model [58], the mice developed a psoriasis-like pathology characterized by massive epidermal hyperproliferation, migration of T cells into the epidermis and an active angiogenic program throughout the dermis as well as at the dermal–epidermal junction. Development of this pathology was associated with an impaired ability of D6-deficient mice to remove inflammatory CC chemokines from the inflamed skin. Similarly, in the model involving subcutaneous CFA injection [59], drainage of inflammatory CC chemokines was impaired in D6-deficient mice, and granulomata developed to a substantially larger size, compared to WT mice. In keeping with the close association between inflammation and cancer [60], we have reported an enhanced tumourigenic programme in D6-deficient mice in models of inflammation-dependent cutaneous tumour development [61]. Thus, in the classic two-stage model of skin tumourigenesis, D6-deficient mice develop almost twice the number of papillomas that WT mice do, and this is associated with an enhanced inflammatory response in the skins of the D6-deficient mice. Interestingly, transgenic expression of D6 in WT mice reduces tumour development, suggesting that the ability of D6 to regulate the overall inflammatory environment controls the extent of tumour development in inflammatory contexts. This conclusion is also supported by clinical studies demonstrating an inverse correlation between D6 expression and disease-free survival in a variety of cancer contexts [62,63]. D6 is also prominently expressed in a range of inflammatory cutaneous pathologies. Thus, in psoriasis [43] and systemic sclerosis [64], elevated D6 expression is seen in both peripheral blood leukocytes and patient skin. In the specific context of psoriasis, D6 is highly expressed in uninvolved psoriatic skin at a level that is approximately eight times that seen in healthy control skin. Importantly, this expression level drops at perilesional and lesional sites, suggesting that D6 expression in uninvolved psoriatic skin may play some role in maintaining histological 'normality' in the presence of a constitutive low-grade inflammatory response. Microtrauma, such as tape-stripping, is sufficient to reduce D6 expression in uninvolved skin, suggesting that such minor tissue insults may trigger the emergence of psoriatic plaques, at least in part by reducing the anti-inflammatory effects of D6. Thus, a reduction in D6 expression may contribute to the 'Koebner phenomenon' seen in certain psoriasis patients [65], in which plaques develop around areas of microtrauma. D6 expression is approximately 10-fold higher on peripheral blood leukocytes of psoriatic or systemic sclerosis patients compared to healthy control leukocytes. In the context of systemic sclerosis [64], leukocyte D6 expression correlates negatively with the circulating levels of inflammatory chemokines, suggesting an active role for peripheral blood leukocyte-expressed D6 in the scavenging of inflammatory chemokines in the circulation of patients with chronic inflammatory pathologies. Importantly, elevated D6 expression is also seen on peripheral blood leukocytes of patients with rheumatoid arthritis (data not shown), suggesting this to be a general phenomenon in chronic inflammatory pathologies.
- *The gut*. In healthy control gut, and in samples obtained from patients with inflammatory bowel disease and colon cancer, the major sites of D6 expression are on lymphatic vessels and leukocytes [66]. Similarly, in mice, D6 is expressed predominantly on stromal cells and B cells of the resting colon [67]. There are contradictory reports in the literature of the effects of D6 deficiency on gut inflammatory responses in the murine DSS colitis model. On the one hand, an unexpectedly reduced susceptibility to colitis, associated with less pronounced clinical symptoms, has been reported in one publication [67], whereas in another, enhanced susceptibility to DSS colitis in D6-deficient mice has been shown [66]. In the publication reporting enhanced susceptibility, radiation chimera studies demonstrated that this is specifically related to the absence of D6 in the stromal/lymphatic compartment and that D6-positive haematopoietic cells in the gut and circulation do not contribute to this phenotype [66]. Notably, and in keeping with the association of D6 with enhanced inflammation-dependent tumourigenesis in skin, in the gut a similar phenotype is also seen in models of colitis-associated cancer, in which D6-deficient mice show increased susceptibility to cancer development [66].
- *The lung*. Initial studies examined the response of D6-deficient mice, compared to WT mice, in the Th2

cell-dependent OVA model of lung inflammation [68]. Using this model, D6 was shown to be important for regulating levels of the Th2 chemokines CCL17 and CCL22, although it appeared to do so only within a certain concentration range. Notably, D6-deficient mice displayed increased numbers of eosinophils, dendritic cells and T cells in the inflamed lung parenchyma compared to WT mice, and also demonstrated reduced airway reactivity. The overall conclusion of the study was that D6-deficient mice displayed opposing effects on inflammation and airway reactivity in this model. In addition, D6-deficient mice have an enhanced susceptibility to murine tuberculosis-induced death [69]. Importantly, this was not associated with alterations in mycobacterial load but resulted from a dysregulated, and multiorgan, systemic inflammatory response. In this model, D6-deficient mice displayed markedly higher levels of a range of inflammatory CC-chemokines and pro-inflammatory cytokines in the serum. Most recently, studies on patients with chronic obstructive pulmonary disease have shown them to have significantly increased numbers of D6-positive alveolar macrophages compared to smoking, and healthy, controls [70]. Further correlative analyses indicate that D6 expression was positively related to markers of immune activation and negatively to markers of lung function.

- *Placentation*. The expression of D6 on the syncytiotrophoblast layer of the placenta positions it immediately at the interface between the mother and fetus. Accordingly, a role has been hypothesized for D6 as a suppressor of inflammatory communication between mother and fetus. In keeping with this model, D6-deficient mice display enhanced susceptibility to inflammation-dependent, and autoantibody driven, miscarriage [40]. In addition, studies have shown that, in murine embryo transfer models, syngeneic or semi-allogeneic fetal survival did not require D6 expression in the absence of inflammatory responses. However, D6 does appear to be important for blocking fetal resorption following embryo transfer into fully allogeneic recipients [39].
- *Other pathological contexts*. Further studies have demonstrated an important role for D6 in the regulation of the hepatic inflammatory response. Indeed, there is only a single report associating a D6 single nucleotide polymorphism with disease susceptibility [71], which specifically relates to the magnitude of the inflammatory response following HCV infection [72]. Roles in regulating hepatic inflammatory responses are also supported by observations reporting enhanced susceptibility of D6-deficient mice in murine models of liver inflammation [72]. Finally, emerging studies suggest a significant role for D6 in the regulation of cardiovascular disease [73]. Specifically, D6-deficient mice appear to be susceptible to excessive inflammation and adverse ventricular remodelling following myocardial infarction. Again, in this context, bone marrow radiation chimera studies indicated that this phenotype was related to radiation-insensitive, presumably non-leukocytic, cell types.

D6 and the regulation of the *in vivo* immune response

Expression of D6 on lymphatic endothelial cells means that it is also positioned at another important biological barrier, ie that between the innate and adaptive immune responses. Interestingly, studies examining the responsiveness of D6-deficient mice in the antigen-driven experimental autoimmune encephalitis model of multiple sclerosis revealed, somewhat counter-intuitively, that D6-deficient mice were less susceptible than WT mice to encephalitis in this model [74]. This was associated with reduced T cell priming and with retention of clusters of dendritic cells within the dermis at the site of injection of the 'mog' antigen and CFA, which are required for initiation of the encephalitic immune response. This phenotype can be accounted for by considering the role of D6 on lymphatic endothelium [75,76]. At this cellular site, D6 appears to be primarily responsible for ensuring that inflammatory CC chemokines do not adhere to lymphatic endothelial surfaces and therefore do not precipitate inappropriate inflammatory cell recruitment to the lymphatic system. This therefore ensures selective presentation of homeostatic CC-chemokines and preferential migration of CCR7-positive antigen-presenting cells to lymph nodes [7]. In D6-deficient mice, in the context of inflammation, lymphatic endothelial cell surfaces become 'shrouded' with inflammatory CC chemokines, resulting in massive inflammatory leukocyte association with this important cell surface. This reduces the efficiency of antigen-presenting cell movement from peripheral sites and, indeed, reduces the flow of lymph, and soluble antigen, to lymph nodes. Together this has the effect of impairing antigen presentation and suggests that lymphatic endothelial cell D6 is an important coordinator of innate and adaptive immune responses.

In a further study, D6-deficient mice were shown to have increased numbers of $Ly6C^{high}$ monocytic cells in the blood and secondary lymphoid organs [77]. These cells appear to have enhanced immunosuppressive activities and are capable of inhibiting the development of adaptive immune responses. This suggests that, in D6-deficient mice, adaptive immunity may be inhibited on at least two different levels. Furthermore, and in keeping with the reduced development of adaptive immune responses in D6-deficient mice, these mice are partially protected from graft-versus-host-disease. D6 has also been implicated in cardiac allograft rejection [78], as increased D6 expression is associated with the severity of allograft rejection and, within grafts, the major site of D6 expression appears to be $CD45^{+}CD68^{+}$ leukocytes. Exactly how D6 is functioning in this pathological setting is not yet apparent.

How does D6 function to regulate the inflammatory response?

It is clear from the literature reviewed above that D6 is an important regulator of the resolution of tissue-specific inflammatory responses. However, the precise mechanism by which it does this is currently a matter of some debate. The typical D6-expressing cell types at an inflamed site, mainly lymphatic endothelial cells, are unlikely to be able to efficiently scavenge chemokines throughout such a site, suggesting that this may not be the most accurate model to describe D6 function in inflammation [75]. Furthermore, a number of the signalling inflammatory chemokine receptors are also able to scavenge their ligands [79] and, as these receptors will be carried on their expressing cells to the epicentre of inflammatory responses, these may have more importance for the direct scavenging of chemokines at inflamed sites than D6. Nevertheless, D6 is clearly important for enhancing the removal of inflammatory chemokines from inflamed sites. One model to explain this relates to the role for D6 on lymphatic endothelial cells [76]. Specifically, the enhanced association of inflammatory leukocytes with inflamed D6-deficient lymphatic endothelium, and the reduced lymph flow from peripheral tissues to lymph nodes discussed above, is associated with impaired chemokine and cytokine clearance from inflamed sites. It may be, therefore, that D6 functions mainly to ensure the 'openness' of lymphatic drainage channels from inflamed tissues and in this way contributes to the resolution of inflammation by enhancing chemokine drainage from inflamed sites.

Conclusions

Identification and characterization of the members of the 'atypical' chemokine receptor family has revolutionized our understanding of the overall control of chemokine function in the orchestration of the *in vivo* inflammatory response. In particular, D6 presents itself as an essential regulator of inflammatory CC chemokine function, and a number of studies have now highlighted D6 as an important contributor in a range of pathological contexts. A major challenge for the future will therefore be to try to use our emerging understanding of D6 biology in a variety of therapeutic contexts. In a similar way, as our understanding of the other 'atypical' chemokine receptors evolves, their potential relevance to pathology and therapy will become apparent.

Author contributions

GJG and ML contributed equally to the writing, manuscript editing and submission of this review.

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