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Review Article

Immunomodulatory properties of CNF1 toxin from *E. coli*: implications for colorectal carcinogenesis

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Abstract: Colorectal cancer (CRC) is a leading cause of cancer death worldwide. The risk of developing CRC is influenced by both environmental and genetic factors. Recently, chronic inflammation and gut microbiota modifications have been associated with increased CRC risk. *Escherichia coli* belongs to the commensal intestinal flora and can become highly pathogenic following the acquisition of genes coding for virulence factors, such as the cytotoxic necrotizing factor type 1 (CNF1). Numerous reports highlight that, besides exerting direct effects on epithelial cells, CNF1 can also act on immune cells, modulating their responses and possibly contributing to disease development. In the present review, we summarized the key studies addressing the immunomodulatory functions of CNF1 and discussed the contribution that CNF1 can bring about to CRC through the creation of a pro-inflammatory microenvironment.

Keywords: CNF1, *Escherichia coli*, carcinogenesis, gut microbiota, immune system, inflammation, colorectal cancer, bacterial toxins

Introduction

Colorectal cancer (CRC) is a relevant public health problem and a leading cause of cancer death worldwide, with more than 900,000 estimated deaths across the globe in 2020 [1]. CRC has a multifactorial etiology, with hereditary factors accounting for approximately 35% of CRC risk [2]. The majority of CRC cases arise as a consequence of ageing, unhealthy lifestyles and/or persistent infections. Chronic inflammation and gut microbiota modifications have also been associated with increased CRC risk [3]. Patients suffering from inflammatory bowel disease (IBD), including young age patients, have an increased risk of CRC compared to healthy subjects [4-6]. It has been proposed that long-standing chronic inflammation initiates and drives tumorigenesis through the induction of oxidative stress, epithelial cell proliferation, and angiogenesis [7]. On the other hand, chronic inflammation can also promote changes in the gut microbiota composition, which in turn, perturb intestinal immunity and tissue homeostasis, also contributing to tumor development.

Escherichia coli represents one of the major inhabitants of the intestine. Although *E. coli* belongs to the commensal gut microflora, it can become highly pathogenic following the acquisition of genes coding for virulence factors. Thus, pathogenic *E. coli* can escape host defences and colonize extra-intestinal sites, causing tissue damage and diseases ranging from urinary tract infections to life-threatening bacteraemia [8]. A high number of virulence factors have been ascribed to *E. coli* and, among the others, the cytotoxic necrotizing factor type 1 (CNF1), whose gene was found in some cases of cancer-associated *E. coli* [9, 10]. A number of reports highlight that, besides exerting direct effects on epithelial cells, CNF1 can also modulate the host's immune response, possibly contributing to CRC development. In the present review, we will provide an overview of the

immune-mediated effects of CNF1 and discuss their possible role in CRC pathogenesis and progression.

Mechanisms of CRC pathogenesis

CRC arises in the colon or rectum portion of the large intestine. It develops slowly, over years and through a multistage process resulting from the accumulation of genetic mutations. Only a minority of CRC cases are genetically inherited. Among non-hereditary CRCs, around 60-90% of cases arise via the adenoma-carcinoma pathway. In this pathway, CRC begins with the formation of benign adenomas, which may subsequently progress to dysplastic adenomas and, in the end, to colonic carcinomas [11]. An alternative pathway to malignancy is represented by the serrated polyps pathway, in which hyperplastic polyps progress to serrated neoplasms and, a fraction of these, culminate in CRCs [11]. Cancers that derive from the adenoma-carcinoma pathway are characterized by chromosomal instability (CIN), mutations in oncogenes (i.e., KRAS) and in tumor suppressor genes (e.g., APC, p53). Cancers deriving from serrated polyps display exceptionally high frequency of aberrantly methylated CpG dinucleotides. Aberrant immunologic signaling pathways and pro-inflammatory mediators also contribute to the transition from pre-cancerous lesions to CRC [12]. In addition, accumulating evidence suggests that gut microbiota disorders (i.e., dysbiosis) are a key environmental factor implicated in the development of CRC, due to the ability of gut microbiota to modulate changes in innate immunity and inflammation.

The host-microbiota interplay in CRC development

The gut microbiota comprises more than 10^{14} microorganisms, which contribute to the regulation of a range of physiological and pathological processes, including cancer development. In the healthy gut, microbiota plays a crucial role in the maintenance of tissue homeostasis, preventing enteric pathogen colonization [13] and regulating mucosal immune responses [14]. Under certain circumstances, a dysbiosis occurs changing the composition of gut colonizers with a major impact on intestinal homeostasis. Dysbiosis of gut microbiota is reported in many human chronic immune-mediated diseases, such as inflammatory bowel disease (IBD) [15] and is associated with a large number of malignancies, including CRC (reviewd in [16, 17]). It is estimated that chronic inflammation likely due to a dysregulated gut microbiota, contributes to approximately 20% of all CRC cases [18]. Although the precise mechanisms by which microbiota induces chronic inflammation and increases CRC risk have not been fully elucidated, some events are thought to contribute to inflammation-driven CRC. As a consequence of pathogenic bacteria-induced epithelial barrier disruption, myeloid cells residing in the lamina propria become activated and release reactive oxygen species (ROS) and reactive nitrogen species (RNS) into the surrounding tissue contributing to DNA damage, chromosomal instability and epigenetic silencing of tumor suppressor genes [19]. In addition, activated myeloid cells produce large amounts of inflammatory cytokines (e.g., IL-6, IL-1, IL-23, IL-17) and immunosuppressive factors (e.g., nitric oxide, arginase, IL-10), which further contribute to the creation of a microenvironment favourable to neoplastic transformation and tumor growth [20]. Of note, long-lasting chronic inflammation can, in turn, promote changes in the gut microbiota composition. In patients suffering from IBD [21] and in a model of colitis-associated CRC [22] a higher abundance of *Enterobacteriaceae/E. coli* was observed in the intestinal microbiota. Along these lines, patients with IBD and CRC displayed an increased prevalence of mucosa-associated *E. coli* compared to control healthy subjects [23, 24]. This evidence highlights the existence of a dynamic interplay between intestinal immunity and gut microbiota whose dysregulation is involved in neoplastic transformation.

The role of bacterial toxins in CRC pathogenesis

Although numerous reports have focused on how dysbiosis leads to CRC, the role of bacterial toxins in cancer development and progression is receiving increasing interest. Besides the induction of inflammation, enteric bacteria and their toxicogenic products may initiate CRC by inducing epithelial DNA damage or dysregulation of key cellular pathways involved in proliferation, apoptosis, differentiation and cell motility. Dysregulation of these pathways has been associated with carcinogenesis and tumor progression [25, 26]. In addition, some

microbial factors contribute to carcinogenesis by triggering oxidative stress, changes in the stem cell niche of epithelial cells or modulation of the microenvironment in a way that regulates the host immune response [27, 28]. One of the most studied toxins is Colibactin produced by *E. coli* (CoPEC) strains, which induces chromosomal instability and DNA damage in eukaryotic cells, leading to senescence of epithelial cells and apoptosis of immune cells [29]. Of note, intestinal inflammation appears essential for CoPEC-driven carcinogenesis, as these bacteria failed to induce CRC in inflammation-free mice [30]. Very recently, Pleguezuelos-Manzano and coworkers [31] demonstrated that a prolonged exposure of a human intestinal organoid to CoPEC originated the same mutational signature detected in a subset of human cancer genomes, strongly indicating a possible role for colibactin in CRC. Interestingly, CoPEC are over-represented in biopsies isolated from CRC patients and have been shown to increase the number of tumours in diverse CRC mouse models [29]. Other bacterial toxins have been indicated as CRC inducers (for a review see [25]) based on their mechanism of action or on their ability to promote carcinogenesis in mice. Among the others, the most studied are represented by the *Bacteroides Fragilis* toxin (BFT) produced by enterotoxigenic strains of *B. fragilis* (ETBF), the cytolethal distending toxin (CDT), the cycle-inhibiting factor (CIF) and the CNF1 produced by *E. coli*. Very recently, in a multicenter case-control study with more than 300 biopsy specimens from people undergoing colonoscopy, the correlation between the presence of bacterial genes coding for Colibactin, CDT, CIF, CNF1 and BFT toxins and the different stages of CRC was analyzed. The results obtained demonstrated that the CIF toxin is associated with polyps or adenomas, thus indicating a role for this toxin in the early stages of carcinogenesis. On the other hand, Colibactin seems to be a predisposing element for CRC, and toxins from *E. coli* as a group have a higher incidence rate in adenocarcinoma compared to controls, paving the way for further insights into the association of *E. coli* toxins with cancerous lesions [10]. Interestingly, these results were obtained irrespectively of the presence of CRC-risk loci.

CNF1 structure and cellular activity

Although belonging to the human intestinal flora, *E. coli* gains pathogenicity following the acquisition of virulence factors, one of which is CNF1. CNF1-producing *E. coli* have been isolated from either intestinal or extra-intestinal infections such as urinary tract infections, bacteraemia and meningitis in neonates [32]. The *cnf1* gene is encoded within the same pathogenicity island of a-Hemolysin (HlyA) and CNF1 and HlyA are always co-transcribed under the same regulator [33], cooperating to favor the persistence of pathogenic bacteria during bacteriemia [34]. How CNF1 is released from bacteria is still unknown.

From a structural point of view, CNF1 is a single chain polypeptide consisting of a binding domain located at the N-terminal end and a catalytic domain at the C-terminus. It binds to host cell surface p67 laminin receptor (p67/LR) and Lutheran (Lu) blood group and basal cell adhesion molecule (BCAM) (Lu/BCAM) [35, 36] and enters endosomes, where the catalytic domain is translocated across the vesicle membrane and released into the cytosol [37, 38] where it exerts its enzymatic activity. The cellular target of CNF1 is represented by the Rho-GTPases Rho, Rac, and Cdc42, crucial regulatory molecules involved in the control of the actin cytoskeleton as well as of a huge number of other cellular functions such as gene transcription, cell cycle progression and a variety of enzymatic activities [39]. CNF1 deamidates the Rho-GTPases at a specific glutamine residue in the switch II region, a region that plays an important role in their GTP hydrolyzing activity [40, 41]. By modifying glutamine into glutamic acid, CNF1 maintains the G proteins in their GTP-bound active form allowing the permanent activation of their effectors. CNF1 activation of Rho proteins is, however, attenuated by a concomitant decrease of their cellular levels via the ubiquitin-mediated proteasomal system [42]. The first evident signs of CNF1 activity is the profound reorganization of the actin cytoskeleton into prominent stress fibers, ruffles and filopodia that impairs cytodieresis and induces multinucleation in several cell types [32]. The activity on the actin cytoskeleton gives rise to a number of actin-dependent cellular activities, such as cell spreading and increase in adhesion properties that are capable, in turn, to hinder apoptosis [43, 44]. In addition, CNF1-treated epithelial cells acquire phagocytic-like activities, being capable to engulf and digest apoptotic cells, non-invasive

bacteria and latex beads [45, 46]. Not least, by regulating the actin cytoskeleton, CNF1 causes cell junction disruption and enhances cellular motility in epithelial cells [42].

Being Rho-GTPases master regulators of a plethora of cellular activities, their activation by CNF1 stimulates cell functions not directly linked to the actin cytoskeleton and that vary depending on the cell context. Activation of transcription factors, cell cycle modulation and inflammatory mediators' expression [45, 47, 48] are some of the CNF1-induced effects reported so far. In this context, we recently reported that CNF1 induces epithelial mesenchymal transition (EMT) in transformed epithelial cells, which is a crucial step in malignant tumor progression and invasiveness. This event occurs by up-regulation of the transcription factors ZEB1 and Snail1, de-localization of the adhesion molecules E-cadherin and β-catenin, mTOR activation, and accelerated invasion and wound healing [49].

Different cell types, including immune cells, have been utilized as recipients for CNF1 [50], but the majority of the studies have been conducted on epithelial cells, as these cells are the main target of the toxin. It is interesting to note that several activities induced by CNF1 in these cells, such as induction of cell motility, protection from apoptosis, activation of pro-carcinogenic signaling pathways and stimulation of inflammatory mediators, are reminiscent of a cancerous phenotype. It is reasonable to think that in complex systems (e.g., the intestinal tissue) the CNF1 produced by *E. coli* not only acts on epithelial cells, but also on the adjacent immune cells, thus orchestrating a complex loop of modifications possibly contributing to carcinogenesis.

Effects of CNF1 on the immune system

Two decades of investigation on bacterial toxins have highlighted the possibility that these virulence factors, beyond causing direct tissue damage to enable pathogen spread, can modulate host immune responses actively contributing to the balance between immunosurveillance and immunopathology [51]. The first evidence of a direct effect of CNF1 on immune cells dates back to the late 90 s. Using primary human monocytes and a myelomonocytic cell line, Capo and colleagues evidenced that CNF1 toxin induces dramatic morphologic alterations and increased filamentous actin content in the cells, negatively affecting integrin-mediated phagocytic activity [52]. Likewise, in another study, treatment of blood polymorphonuclear leukocytes (PMNL) with CNF1 induced remodelling of the actin cytoskeleton and the stimulation of oxygen radical production, an increased adherence of PMNL on epithelial cells and a decreased bacteria phagocytosis [53]. Cytoskeleton rearrangements are not only important for cellular motility and phagocytosis, but also for the dynamic processes supporting the cytotoxic activity of immune cells. In fact, treatment of NK cells with CNF1 induced a transient activation of the Rac-GTPase which, in turn, orchestrated the cytoskeleton arrangements needed for the formation of the immunological synapse and for killing activity [54]. Also, treatment with CNF1 increased the surface expression of molecules involved in cell activation (e.g., CD69 and HLA-DR), of some adhesion molecules involved in NK/target cell pairing (e.g., CD18 and ICAM-1) and, to a lesser but significant extent, IL-2R (CD25) [54]. More recently, CNF1 has been reported to trigger the phenotypic and functional maturation of immature human monocyte-derived DCs (mo-DCs). Culture of mo-DC with CNF1, but not with its enzymatically inactive control, induced the up-regulation of CD86 and CD83 maturation markers, the secretion of IL-6 and TNFα and the proliferation of allogeneic CD4+T cells, indicating their full functional competence [55].

The intrinsic immunostimulatory capacity of CNF1 was deeply investigated by Boyer and colleagues in *Drosophila melanogaster*. In this model, CNF1 proved sufficient to induce an immune response in the absence of other microbial-derived stimuli. However, the immune response to CNF1 was initiated not by direct recognition, but in response to activation of a host protein, the Rho-GTPase Rac2 which, in turn, engaged immune signaling pathways via the innate immune adaptors IMD and Rip1-Rip2 in flies and mammalian cells, respectively [56]. Confocal microscopy analysis of murine bone-marrow-derived macrophages (BMDMs) treated with CNF1 revealed that NLRP3 inflammasome is the major sensor involved in the CNF1-triggered Caspase-1 activation and pro-inflammatory cytokine secretion [57]. Inflammasomes are cytoplasmic multimeric complex-

es present in many cell types, including myeloid and epithelial cells, that form upon sensing of a diverse range of pathogen-associated molecular patterns and danger-associated molecular patterns [58]. The final common pathway of inflammasome signaling is inflammatory caspase-activation and IL-1β and/or IL-18 production. In CNF1-exposed BMDMs the NLRP3 inflammasome is activated by a signaling cascade involving the P21 activated kinases (Pak) 1/2 ultimately leading to IL-1β cytokine maturation [57]. Interestingly, treatment of BMDM with CNF1 did not affect IL-6 or TNF-α secretion, two cytokines that are not regulated by inflammasomes. These studies highlight that mammalian innate immune system has evolved strategies to detect abnormal activation of Rho-GTPases occurring as a consequence of some pathogenic bacterial infections. In a mouse model of bacteraemia induced by CNF1-producing *E. coli*, IL-1β-mediated host responses promoted an efficient destruction of bacteria and host survival [34]. In addition, mice infected with CNF1-producing *E. coli* showed increased circulating Gr1+ CD45+ cells compared to mice infected with CNF1-defective *E. coli* suggesting an active role of CNF1 in the increased recruitment of Gr1+ cells [34]. It has been proposed that the abnormal activation of Rac/Cdc42-GTPases by CNF1 triggers the assembly of an anti-virulence immune complex involving NOD1 and RIP kinases to promote NF-κB activation and the secretion of proinflammatory cytokines (e.g., IL-1β) which, in turn, mobilize Gr1+ cells [34]. It is worth noting that CNF1 has also been reported to induce the secretion of inflammatory factors from endothelial and epithelial cell lines [45, 59]. This evidence implies that several cell types may contribute to CNF1-induced inflammation in peripheral tissues.

CNF1-producing uropathogenic *E. coli* (UPEC) elicits significantly more interstitial and submucosal edema and neutrophil infiltration in the bladder than an isogenic CNF1-negative strain, although both strains colonize the urinary tract equivalently [60]. Microarray analyses of host RNA isolated from the bladders of mice challenged with various UPEC identified numerous altered cellular pathways and increased expression of genes that encode pro-inflammatory cytokines, regardless of the bacterial strain [60]. CNF1-producing *E. coli* specifically upregulated the transcription of genes involved in the innate immune response (e.g., MARCO) and had a negative effect on metabolism- and transport-associated genes [60]. In a pyelonephritis mouse model, Huan Yang and colleagues found that CNF1 increased neutrophils and decreased bacterial clearance in infected bladder and kidney tissues by inhibiting non-opsonic macrophage phagocytosis through the downregulation of CD36 transcription [61].

Despite the advances in the understanding of the complex interplay between GTPases and immune responses, the role of CNF1 in the regulation of the anti-tumor cytotoxic response remains largely unknown. CNF1 induces T lymphocyte functional changes by activation of the GTP-binding protein Rho. It has been proposed that, during acute colitis due to specific *E. coli* strains, CNF1 toxin could act on T lymphocytes by increasing their adherence to the intestinal epithelial cells [62]. Also, by stimulating TNF-α and TGF-β production by T lymphocytes, CNF1 may in turn amplify the inflammatory consequences of leucocyte trans-epithelial migration in response to the bacterial colonization [62]. Evidence exists that CNF1 can affect not only the morphology and function of target cells, but also their immunovisibility. In one study, treatment of the resistant non-small cell lung carcinoma cell line IGR-HeuR8 with CNF1 restored the parental morphology and mitigated the cell resistance to CTL-mediated killing [63].

Some findings suggest that CNF1 may mediate its effects in the absence of direct bacteria/host contact. One report shows that biologically active CNF1 from UPEC is delivered to PMNs via extracellular vesicles (EV) and that these complexes can alter the chemotactic function of PMN cells [64]. Along these lines, in another study CNF1 delivered by EVs released from CNF1-exposed epithelial cells induced cytoskeleton changes and activation of Rac1 and NF-κB in target cells in a way comparable to that triggered by soluble CNF1 [65]. These observations strongly support the hypothesis that EVs can offer CNF1 a route to travel from cell to cell.

The ensemble of these studies highlights the variety of effects that CNF1 can exert on immune cells and suggest the need of further investigation on the mechanisms underlying

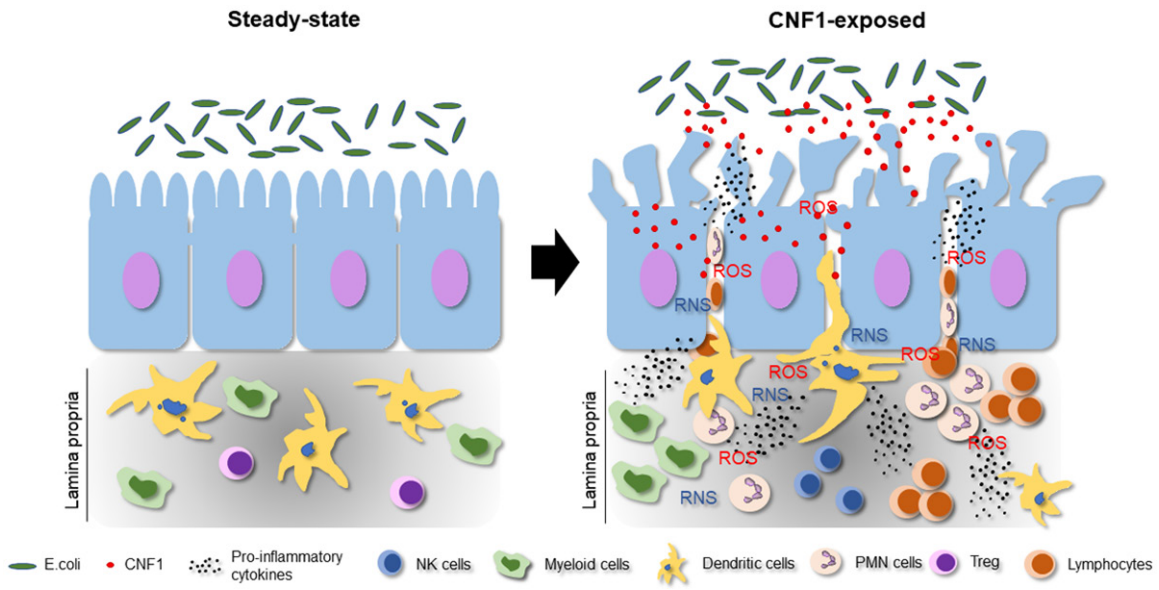

Figure 1. Schematic representation of the putative in vivo effects of CNF1. Following exposure to CNF1-producing *E. coli*, epithelial cells are modified in their actin cytoskeletal organization and cell-cell junctions. The resulting change in the permeability of the intestinal epithelium allows CNF1 to reach the underlying lamina propria and interact with resident myeloid cells. The direct activation of Rho-GTPases in immune cells by CNF1 induce secretion of ROS and RNS as well as pro-inflammatory cytokines, which, in turn, recruit neutrophils, lymphocytes and NK cells. These events lead to the generation of a chronic inflammatory state contributing to CRC development.

the complex interplay between gut bacteria and the host tissue.

Outlook and conclusions

Accumulating evidence suggests that several bacterial toxins exert tumorigenic effects either by directly damaging DNA or, as for CNF1, by dysregulating key eukaryotic processes, such as cellular signalling and cell death. The majority of studies on the effects of CNF1 have been conducted on epithelial cells, being this cell type the immediate target of bacterial toxins. However, these studies have been mostly carried out *in vitro*, disregarding the interplay between epithelial cells and their microenvironment. Few studies have addressed the effects of CNF1 on immune cells, although evidence exists on a role of Rho-GTPases in thymocyte development and peripheral T cell homeostasis [66]. In colon carcinogenesis, chronic inflammation triggered by infections markedly increases the risk of tumour development [67]. In addition to the evidence reviewed herein on the direct effects of CNF1 on immune cells, CNF1 can indirectly contribute to the creation of a pro-inflammatory microenvironment by inducing: i) a rise in cell monolayer paracellular permeability allowing the trans-epithelial migration of PMN cells and/or bacteria or bacterial products; ii) the production of pro-inflammatory factors stimulating the recruitment of pro-inflammatory immune cell subtypes (**Figure 1**). All these phenomena, together with the reported pro-angiogenic effects [68], could contribute to CRC development. In this respect, it is interesting to note that changes in the microenvironment are reported to be at the basis of the promotion of colon tumor growth by the bacterial toxin Colibactin [69].

On the other hand, beneficial effects for CNF1 in animal models of Alzheimer's disease [70] Rett syndrome [71], and murine glioma [72] have been reported. CNF1 has also been proposed as mucosal immunoadjuvant in prophylactic and therapeutic vaccination against diverse diseases [73-75]. These apparently conflicting evidences suggest that the consequences of the biological effects of CNF1 may be dependent on the anatomical site, on the type of cell target and on pre-existing inflammatory conditions. In this context, recent evidences indicate that CNF1 may induce EMT in already transformed epithelial cells, but not in normal cells, unless they are exposed to an

inflammatory environment [49]. At the same time, CNF1 reinforces the supportive activity of astrocytes [76] while causing senescence and death in astroglioma cells [72].

In conclusion, further studies are needed to clarify the causal link between CNF1 and CRC and the effects of CNF1 on the intestinal microenvironment. Since microbiota alterations have been reported in CRC [16], specific bacteria-associated biomarkers, such as CNF1, might be used in the future, eventually in concert with chronic inflammation markers, to redefine risk categories and to design novel preventive strategies against CRC.

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Disclosure of conflict of interest

None.

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