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Cystatin protease inhibitors and immune functions

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TABLE OF CONTENTS

1. ABSTRACT

Cystatins are natural tight-binding reversible inhibitors of cysteine proteases. They are wide spread in all living organisms (mammals, nematodes, arthropods etc.) and are involved in various biological processes where they regulate normal proteolysis and also take part in disease pathology. Many cystatins show changes in expression and/or localization, as well as changes in secretion, following certain stimuli acting on immune cells. In immune cells, cystatins interfere with antigen processing and presentation, phagocytosis, expression of cytokines and nitric oxide and these ways modify the immune response. Further, it has been suggested that cystatin-type molecules secreted from parasites down-modulate the host immune response. Precise understanding of the regulatory roles on proteolytic enzymes of endogenous and exogenous cystatins, such as those from parasites, will provide us with valuable insight into how immune response could be modulated to treat a specific disease. This review covers some specific functions of individual cystatins, with a particular focus on the relevance of cystatins to the immune response.

2. INTRODUCTION

Cystatins are natural tight-binding reversible inhibitors of cysteine proteases (1). The name "cystatins" was proposed (2) to emphasize their function as inhibitors of this particular group of proteolytic enzymes. Cysteine proteases are wide spread in all living organisms (mammals, birds, fish, insects, plants, protozoa, etc.) and are involved in a variety of biological processes. Cystatins have the capacity to regulate normal proteolysis (protein turnover, proprotein processing, bone remodeling, antigen presentation…), as well as taking part in disease progression when misregulated (cancer metastasis, inflammation…). The control of their proteolytic activity by endogenous protease inhibitors is therefore essential to the life of a cell. Besides inhibiting and regulating lysosomal cathepsins, human cystatins inhibit C1 proteases from microorganisms and parasites and can participate in defense against microbial infections. Not surprisingly, cystatins have been found among lipopolysaccharide (LPS)-inducible gene products (3, 4).

In 1968 chicken egg white cystatin (chicken

cystatin), the prototypical type 2 cystatin, was discovered (5) as an inhibitor of ficin and papain. Chicken cystatin was further characterized (6, 7) and its 3D structure (8) revealed structural characteristics explaining its mechanism of inhibition. The first prototypical type 1 cystatin - named stefin - was isolated (9) and characterized (7, 10, 11) from the cytosol of human polymorphonuclear granulocytes. Furthermore, in 1984 the first human homologue of chicken cystatin, human cystatin C, was isolated from the serum of patients with autoimmune diseases (12). Since then knowledge about this family of similar proteins with inhibitory properties has expanded greatly. Many new members have been introduced and their different regulatory roles proposed and established. Nowadays, human stefin and chicken cystatin relatives constitute a large group of evolutionarily related proteins, many of them acting as functional cysteine protease inhibitors in humans. Consequently, novel anti-cancer strategies based on cystatin function have been introduced (13, 14, 15). Although it is well-established by *in vitro* studies that cystatins are potent inhibitors of cysteine proteases (16, 17, 18) their *in vivo* functions are less clear. The present review covers some of these specific functions of individual cystatins, including those relevant to the immune response.

3. OVERVIEW OF THE CYSTATIN FAMILY

The "cystatin family" of proteins derives from a common ancestor and comprises a diverse group of proteins with more or less restricted inhibition profile towards target enzymes. Mutagenesis and X-ray crystallographic studies have revealed three conserved regions which together form a wedge-shaped cystatin structure that blocks the active site of C1 cysteine proteases (1, 8, 19). These regions include an N-terminal glycine, a glutamine–valine–glycine loop segment, and a second C-terminal hairpin loop containing proline and tryptophan residues. As shown in cystatin C, the affinity and specificity of cystatin/cysteine protease interaction also depend upon the presence of the N-terminal region (20). Furthermore, the crystal structure of another cystatin-type inhibitor, stefin A, in complex with cathepsin H explained the interactions between exopeptidases and their cystatin-type inhibitors. It has been shown that the N-terminal residues of stefin A adapt to the active sites of endo- and exopeptidases (21).

Some cystatin-type inhibitors also inhibit asparaginyl endopeptidase (AEP) (22, 23), a mammalian legumain-like protease from the C13 family of cysteine proteases specific for the hydrolysis of the peptide bond following asparaginyl residues. Human cystatins C, E and F and the parasite cystatin homologue *Bm*-CPI-2, all type 2 cystatins, inhibit AEP through a reactive site distinct from that involved in the inhibition of C1 cysteine proteases (24), whereas cystatin D, stefins A and B and kininogens do not inhibit it at all due to their different structural characteristics. Inhibition of serine proteases has also been described for some members of the "cystatin family" (25), though its physiological relevance remains to be elucidated.

A MEROPS classification scheme (26) for proteases has been widely accepted for their inhibitors also, as well as for some evolutionarily related gene products which still remain to be characterized as true inhibitors. The cystatins entered under this classification belong to the MEROPS Family I25 of Clan IH, on the basis of their statistically significant amino acid sequence similarities (26). Based on sequence similarities of homologues, the number of cystatin-like segments and the number of disulfide bonds, cystatins can be divided into different types, *(a)* members with one cystatin domain (stefins, cystatins), *(b)* members with two tandem cystatin domains (fetuins and latexins – though both lack inhibitory activity against cysteine proteases) and *(c)* members with three tandem cystatin domains (kininogens) (27). Type 1 cystatins (stefins A and B) are mostly intracellular, whereas type 2 cystatins (C, D, E/M, F, G, S, SN and SA) are mostly extracellular. Type 3 cystatins (kininogens) are intravascular proteins. In addition, fetuins (mammalian fetal serum and bone glycoproteins) constitute another – type 4 – group of cystatin-like molecules. They lack detectable cysteine protease inhibitory activity, whereas all true cystatins are tight-binding inhibitors of C1 cysteine proteases. In contrast, fetuin orthologs from snake venom were reported to inhibit metalloproteases (28).

3.1. Type 1 cystatins (stefins)

The name stefin was associated with the first isolated cytoplasmic type 1 cystatin, nowadays called stefin A or cystatin A (9). To distinguish between predominantly cytoplasmic and predominantly vesicular inhibitors, both with single cystatin domains, the name stefins is hereafter also used for type 1 cystatins. Stefins are mainly intracellular proteins though they can also appear in body fluids (29). They are single-chain polypeptides with molecular mass around 11 kDa, with no disulfide bridges or carbohydrate side chains, and no signal peptide. Homologues of human stefin A (cystatin A) and stefin B (cystatin B) have been found in mouse rat, cat, pig, cow (26) and even invertebrates (leech) (30). Human stefin A is a potent intracellular inhibitor of most C1 proteases. It exhibits a broad inhibition profile but is present mostly in skin (epithelial cells) (31) and blood cells (9). Given its exclusive distribution, stefin A may display a relevant regulatory potential for exogenous proteases from microorganisms and parasites invading the human host and its immune system through the skin. Furthermore, it has been suggested that stefin A has an important function in neonatal skin development and the associated neonatal immune response (32).

Stefin A is expressed at high levels in immune follicular dendritic cells, present within germinal centers of secondary lymphoid organs (33). It has been speculated that it may prevent apoptosis of B-cells with high affinity receptors for the specific antigen present on the surface of follicular dendritic cells (34, 35). Since the transport pathway of stefin A from follicular dendritic cells towards a nearby B cell is unknown (36), the model remains open to question.

Human stefin B is more widely distributed than its homologue stefin A, and is found inside the cytoplasm

of most human cells, whereas increased levels have been described in a variety of malignant tumors (37). In immune cells, stefin B gene expression is increased upon LPS stimulation of human blood monocytes (3). Furthermore, up-regulation of nitric oxide release from IFN-gamma-activated mouse peritoneal macrophages was observed in the presence of stefin B or chicken cystatin (38, 39), which may indicate a further role, independent of its inhibitory ability. Stefin B involvement in the innate immune response in invertebrates has also been studied. It has been shown that stefin B homologue *Tt-cysb* from leech *Theromyzon tessulatum* is up-regulated after bacterial challenge (30). Furthermore, type 1 cystatins were found in the senile plaques of patients with Alzheimer's and Parkinson's disease (40), suggesting that stefins A and B may also be present *in vivo* in a polymeric form in amyloid plaques. Deletion of the mouse cystatin B gene in knockout mice and loss-of-function mutations in the human cystatin B gene result in neurological dysfunction characterized as a form of epilepsy (41, 42). Furthermore, several mutations of the cystatin B gene were described in patients with progressive myoclonus epilepsy, but the exact physiological function of stefin B in this disease is still not clear (43, 44).

3.2. Type 2 cystatins (cystatin C-like cystatins)

Type 2 cystatins are single-chain polypeptides with molecular masses from 13 to 15 kDa. They are synthesized with a signal peptide enabling the transport of mature cystatin into vesicles and the extracellular space where they are present at higher concentrations than those of type 1 cystatins. Mammalian type 2 cystatins possess two conserved disulfide bridges at the C-terminal end. Cystatins C, D, S, SA and SN exhibit approximately 50% sequence homology, whereas cystatin E/M and cystatin F show less homology (30 – 35%) with other type 2 cystatins. However, they all have a typical signal peptide and conserved disulfide bridges. Type 2 cystatins are generally described as non-glycosylated (45). However, there are exceptions, for example, human cystatins F and E/M which are glycosylated. Besides inhibiting C1 proteases, cystatin C is able to inhibit AEP with a second inhibitory segment located on the loop at the C-terminal end of the alpha-helix, opposite to the C1 protease binding site. Furthermore, cystatin C can form a dimer *in vitro* (46), with two molecules interacting by subdomain swapping, forming two identical domains (47, 48). In spite of the fact that the cystatin fold remains conserved, dimer formation causes the destruction of the C1 protease binding site and thereby loss of inhibitory ability against C1, but not C13 proteases such as AEP (24).

3.3. Type 3 cystatins (high- and low-molecular-mass kininogens)

Type 3 cystatins, or kininogens, are multidomain proteins with high molecular mass (60 – 120 kDa) and three tandemly repeated type 2-like cystatin domains (49, 50). Only the second and third cystatin domains are inhibitory to cysteine proteases. Type 3 cystatins are glycosylated proteins and possess eight disulfide bridges (51). Kininogens are expressed in liver and present in blood in μM concentrations (52). Mammalian kininogens, precursor proteins of vasoactive kinins and participants in the blood coagulation cascade, are present intravascularly as well as in synovial and amniotic fluids (53, 54). If single-domain cystatins are the predominant cysteine protease inhibitors in tissues, kininogens constitute the major source of inhibitory capacity in the circulation, thereby providing systemic protection against leaking lysosomal cysteine proteases, as well as against proteases derived from invading microorganisms. Furthermore, kininogens coordinate adaptive immunity through the proteolytic release of bradykinin which act as an endogenous danger signal driving dendritic cell maturation (55).

3.4. Other cystatin-like candidates

A new subgroup of type 2 cystatins (CRES-like proteins) has been proposed (56), with some of the members identified, such as CRES (cystatin-related epididymal spermatogenic protein) (57), CRES2 (57), CRES3 (57), cystatin 8 (58, 59), testatin (cystatin 9) (60), cystatin 11 (61), cystatin T (cystatin 12) (62), CLM (bone marrow-derived cystatin-like molecule, cystatin 13) (63), SPP-24 (secreted phosphoprotein, cystatin 14) (64, 65), cystatin SC (66), cystatin TE-1 (66), CYMG1 (67, 68) and others. They possess a cystatin domain, but they lack the critical consensus sites that are important for inhibiting cysteine proteases. They are expressed primarily in the male and female reproductive tracts and neuroendocrine tissues (57). For example, CRES does not inhibit cysteine protease papain or cathepsin B, indicating that it probably does not function as a typical cystatin. However, CRES has been shown to selectively inhibit serine protease prohormone convertase 2 involved in prohormone processing in the neuroendocrine system (69). These cystatin-like proteins may have evolved to perform tissue-specific functions, but they need to be further characterized to establish whether they act as biologically relevant protease inhibitors.

4. SPECIFIC FUNCTIONS OF TYPE 2 CYSTATINS

4.1. Cystatin C

4.1.1. Cystatin C and disease pathology

The cystatin C gene is a house-keeping gene and its expression, as well as its protein (originally called gamma-trace) (70) level, has been determined in a wide variety of human tissues, including cell lines and body fluids (29, 70): from seminal plasma (more than 3 μM), tears, cerebrospinal fluid, milk, synovial fluid, urine, blood plasma, saliva to amniotic fluid (less than 0.1 μM). Its abundant and broad distribution together with its ability to bind tightly to target cysteine proteases (17) supports it as an emergency inhibitor, trapping and neutralizing redundant proteolytic activity outside cells (71). It has been suggested that cystatin C regulates the degradation of bone matrix by cathepsin K, both extracellularly and intracellularly (72). Its diagnostic value and prognostic significance have been reported for several diseases (37, 73, 74, 75, 76, 77, 78). Furthermore, correlations have been found between cystatin C expression, mutations, and clinical status of patients with autoimmune disease, cerebral amyloid angiopathy (79) and hereditary brain hemorrhage (80), atherosclerosis and aortic aneurysms

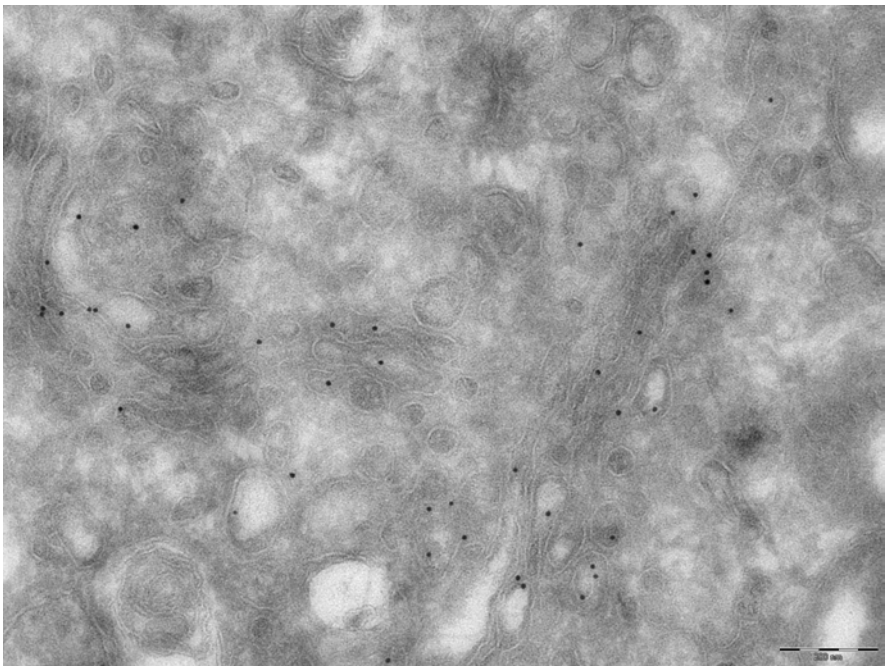

Figure 1. Immunogold-labeled endogenous inhibitor cystatin C, as present in human immature dendritic cells. Ultrathin cryo sections were prepared from fixed dendritic cells and labeled with specific rabbit anti-human cystatin C antibody and Protein A-gold (10 nm). Under the transmission electron microscope 10 nm colloidal gold particles, bound to specific probe (antibody), pointed the exact localization of labeled protease inhibitor. Strong labeling of Golgi apparatus has been observed. Original magnification, ×17500. The scale bar represents 200 nm.

(81), multiple sclerosis (82) and liver dysfunction (83). Amyloid angiopathy is a neurological disease that results from mutations in the cystatin C gene (84, 85, 86). It has been reported that the point mutation in the cystatin C gene, in which Leu68 is substituted for Gln, destabilizes the monomers and increases the stability of the partially unfolded intermediate (47). Furthermore, this cystatin C variant causes formation of amyloid deposits in the cerebral vasculature of patients with hereditary cerebral hemorrhage with amyloidosis – Icelandic type (85). The amyloid deposition in the walls of small- and mid-sized arteries in brain causes the thickening of these arteries, leading to their occlusion and rupture, to brain hemorrhage and, finally, to death in young adults affected by this disease (87, 88).

Cystatin C has also been implicated in other neuronal degenerative diseases, such as Alzheimer disease, though here it does not show amyloid structure (88). Neuronal death of cultured human neurons from the central nervous system has been induced by cystatin C in a dose-dependent manner (89), supporting the involvement of cystatin C in the pathogenesis of Alzheimer's disease. In contrast, overexpression of human cystatin C in mouse models has been reported to reduce cerebral amyloid-beta deposition (90). A protective role of cystatin C in Alzheimer's disease pathology has been proposed (91). Cystatin C has been shown to bind to soluble amyloid-beta peptide and, in this way, to inhibit amyloid-beta deposition and progression of the disease. Modulation of cystatin C concentrations may, therefore, have therapeutic implications for this disease (90, 91).

In another pathological condition, the formation of atherosclerotic lesions, expression of cystatin C is reduced, suggesting a shift in the balance between inhibitors and proteolytic enzymes (elastolytic and collagenolytic cathepsins S, K and L) that favors remodeling of the vascular wall (92). Cystatin C deficiency in apolipoprotein E-deficient mice promotes atherosclerosis (93) and increases elastic lamina degradation and aortic dilatation (94).

The diagnostic value of cystatin C as a marker of kidney dysfunction has also been extensively investigated in clinical studies on adults, children and elderly people. Though many studies show that cystatin C may be a more reliable marker of glomerular filtration rate (GFR) than serum creatinine (95, 96), it is still not routinely used by clinicians. Cystatin C has been studied as a potential marker for relapse in cancer patients with non-Hodgkin B-cell lymphoma (97). The expression of cystatin C gene in immune cells is regulated and is differentiation and maturation dependent (4). On the protein level, a large increase in intracellular cystatin C (see Figure 1) content has been confirmed during differentiation of human monocytes to immature dendritic cells (98). In addition, it has been shown (99, 100) that cystatin C is able to antagonize TGF-beta signaling in normal and cancer cells by interacting with TGF-beta type II receptor, thereby preventing TGF-beta binding. Cystatin C and its derivatives could form the basis for drug development aimed at treating of certain cancers or fibrotic/proliferative diseases regulated by TGF-beta (99). Interestingly, cystatin C binds

to human C4, a component of the classical complement pathway. It has been suggested that this constitutes a regulatory mechanism for complement activation that could be of particular interest at tissue sites where cystatin C and the complement proteins are produced locally by macrophages (101). As for stefin B, up-regulation of nitric oxide release from IFN-gamma-activated mouse peritoneal macrophages was observed in the presence of chicken cystatin (38, 39). In immune cells, cystatin C gene expression is greatly increased on LPS stimulation of human blood monocytes (3). Additionally, cystatin C has been suggested to be a potent regulator in inflammatory processes by regulating the phagocytic function of polymorphonuclear neutrophils (102).

4.1.2. Cystatin C in dendritic cells

Of the professional APC, dendritic cells have the unique capacity to initiate an effective immune response, by stimulating naive T cells. Developmental control of proteolytic activity has been proposed in these cells (103, 104, 105). Initially, based on a study on mouse dendritic cells, cystatin C was suggested to be of major importance, supposedly regulating the proteolytic activity of cathepsin S during maturation of dendritic cells (106). Changes in cathepsin S activity were shown to correlate with a redistribution of MHC class II molecules (MHC II) at the surface of dendritic cells during their activation (maturation), suggesting a role for cystatins in the control of antigen presentation in professional APC (106). Furthermore, the process of cell maturation in human dendritic cells has been found to be accompanied by a significant decrease of cystatin C protein and its markedly changed localization (98, 107), in contrast to the results of Lautwein *et al.* (104), who reported unaffected expression and distribution of cystatin C during this process. The issue has not been completely resolved, and the proposed immunomodulatory role of cystatin C on cathepsins involved in the maturation of MHC II, and the successive degradation of MHC II associated chaperone invariant chain (Ii) to CLIP, remains controversial. This is so, because no defect in MHC II surface expression has been observed in mature dendritic cells from cystatin C-knockout mice (108, 109). Although cystatin C is expressed differentially in different dendritic cell populations, it has not been confirmed that endogenous cystatin C sufficiently controls Ag presentation in mouse dendritic cells (109).

4.2. Cystatin F (leukocystatin)

Cystatin F (110, 111, 112) is a glycoprotein with two glycosylation sites, at the N- and C-terminal ends. In addition to the two disulfide bridges common to all type 2 cystatins, mature cystatin F has two additional Cys residues, which makes cystatin F unique among single-domain cystatins (110). Cystatin F has been found predominantly in hematopoietic cells such as monocytes, dendritic cells and certain types of T-cells and NK cells (110, 111, 112, 113). Cystatin F levels in human tissue and body fluids (blood serum, pleural fluid) are lower (pM) than those of cystatin C (μM) (75). The expression of cystatin F gene is regulated and is differentiation dependent. In dendritic cells cystatin F mRNA levels were significantly upregulated during cell maturation (4). In contrast, high cystatin F protein content and decreased secretion were observed on activation of human pro-monocytic U-937 cells with retinoic acid or tetradecanoyl phorbol acetate (114). Cystatin F trafficking before secretion and its *in vivo* intracellular dimer formation are still imperfectly characterized (115), whereas a crystal structure of cystatin F dimer has been described that explains the structural basis of the reduction-dependent activation of human cystatin F (116). Compared with other cystatins (117), cystatin F exhibits a distinct specificity profile, binding tightly to cathepsins F, K, L and V, less tightly to cathepsins S and H, and not inhibiting cathepsins B, C or X (18). Furthermore, although cystatin F can inhibit AEP, its affinity is less than that of other AEP binding cystatins (24). In U937 cells a protective role of cystatin F, against cathepsins misdirected to specific cystatin F containing vesicles, was proposed on the basis of different inhibition profiles and its intracellular localization (18).

4.3. Cystatin E/M

Cystatin E/M is a secretory glycoprotein with a relatively low similarity to other human type 2 cystatins. It shows 26 – 37% amino acid identity but, in contrast to cystatin C, cystatin E/M is glycosylated at the N-terminal end and is about 100-fold weaker as an inhibitor of cathepsin B (117, 118). In contrast, cystatin E/M is a strong inhibitor of AEP and of cathepsins L and V (119). Expression of cystatin E/M is restricted to epidermis, more precisely to stratum granulosum, sweat glands, sebaceous glands and hair follicles (119). Stratum specific co-localization of cystatin E/M with cathepsin V has been confirmed, supporting a role for cystatin E/M role in epidermal differentiation and desquamation (120, 121). The absence of cystatin E/M causes unrestricted activity of AEP in hair follicles and epidermis, the location where cystatin E/M is normally expressed. In cystatin E/M deficient mice the processing of transglutaminase 3 is increased due to the unrestricted activity of AEP (122). These mice show juvenile lethality and abnormal epidermal cornification, a disease phenotype resembling the human keratinization disorder harlequin ichthyosis. On the other hand, it has also been suggested that cystatin E/M regulates cross-linking of structural proteins by transglutaminase 3 in cornified envelope formation, by inhibiting cathepsins L and V, both involved in processing transglutaminase 3 (119). Silencing cystatin E/M in tumor cells enhanced the invasion, motility and hyperproliferation of tumor cells through a cysteine-protease-dependent pathway, implying that silencing cystatin E/M significantly increased the proteolytic activities of cathepsins B and L and AEP (123).

4.4. Cystatin D

55% of the sequence of cystatin D is identical to that of cystatin C, with all the sequence motifs essential for cysteine protease inhibition being well conserved (124). Compared to other type 2 homologues, cystatin D presents an unusual, more restricted, inhibition profile with preferential inhibition of cathepsin S > cathepsin H > cathepsin L and no inhibition of cathepsin B or pig legumain. Its crystal structure showed that the protease-interacting regions differ from those of other cystatins,

providing an explanation for the restricted inhibitory specificity for some C1 proteases and its lack of reactivity towards legumain-related enzymes (125). Furthermore, and in contrast to its homologue cystatin C, cystatin D shows much more limited localization (saliva, tears) (124). Whether this indicates that cystatin D relevant physiological targets are located in saliva and tears, or that they originate from pathogenic organisms invading the immune system, needs to be further investigated.

4.5. Cystatins S, SA and SN

Human cystatins S, SA and SN are non-glycosylated proteins that display amino acid sequence identities of about 90% in pairwise comparisons, with an overall identity to the cystatin C and cystatin D sequences of about 50%. They are present in saliva, tears, urine, seminal plasma, liver and muscle. The quite specialized glandular localization may support their function as defense inhibitors in secretion, directed towards exogenous cysteine proteases (45). On the other hand, an example of the involvement of salivary type 2 cystatins in the immune response through the cytokine network has been described for cystatin SA stimulation of IFN-gamma production by $CD4^+$ lymphocytes (126).

5. CYSTATINS FROM NEMATODES

Parasites have evolved to invade and survive in their host until they are ready to reproduce. Parasites release a variety of molecules that enable them to penetrate defense barriers and avoid host immune attack. Immunomodulation by nematode cystatins involves interference with antigen presentation and T cell response, and modulation of cytokine production and inducible nitric oxide production, as well as the allergenic potential of some nematode cystatins (127, 128). Cystatins from both parasitic and free-living nematodes differ substantially with regard to their immunomodulatory properties. Cystatins from parasites probably evolved during co-evolution of the parasites with their hosts, to acquire anti-inflammatory properties (127, 129).

Nematode cystatins inhibit proteases involved in antigen processing and presentation within APC, leading to a decreased T cell response. For example, Bm-CPI-2, a cystatin homologue produced by the filarial nematode parasite *Brugia malayi*, inhibits a number of cysteine proteases (cathepsins B, L, S and AEP) inside the endosomes and lysosomes of human B cells. Moreover, Bm-CPI-2 substantially inhibits the presentation of selected T cell epitopes from tetanus toxin (130). A similar role was described for nippocystatin, another type 2 cystatin inhibitor from the intestinal nematode *Nippostrongylus brasiliensis*. It has been shown that secreted nippocystatin modulates antigen processing, helping this parasite to evade the host immune system (131). Nematode cystatins also modulate the cytokine response, the most prominent trait being the up-regulation of Th2 cytokine IL-10 by macrophages, which leads to downregulation of costimulatory molecules on the surface of these APC. This further contributes to the induction of an anti-inflammatory environment, concomitant with a strong inhibition of cellular proliferation which favors the survival of parasites in the host (127). A contrasting proinflammatory activity of nematode cystatins, i.e. upregulation of production of inducible nitric oxide by IFN-gamma-activated macrophages, has been described (38, 39). Furthermore, on comparing the immunomodulatory effects of two cystatins from filarial nematodes *Onchocerca volvulus* and *Acanthocheilonema viteae* with those of two homologous proteins from the free-living nematode *Caenorhabditis elegans*, Schierack *et al.* (132) showed upregulation of inducible nitric oxide in IFN-gamma-stimulated murine macrophages for both groups of nematode cystatins. On the other hand, filarial cystatins suppressed the proliferation of human peripheral blood mononuclear cells and murine spleen cells, while the *C. elegans* cystatins were less effective. Filarial cystatins markedly increased the production of IL-10 by human peripheral blood mononuclear cells, whereas *C. elegans* cystatins increased the production of IL-12 and IFN-gamma.

6. CYSTATINS FROM TICKS

Ticks have developed a series of mechanisms to gain undisturbed access to their nutritious meal, one of them being the secretion of saliva into the biting site. Saliva from the widely distributed tick *Rhipicephalus sanguineus* has been reported to inhibit differentiation and maturation of murine bone-marrow-derived dendritic cells, reducing their production of IL-12 and thus causing them to be poor stimulators of cytokine production by antigen-specific T-cells (133). Furthermore, sialostatin L (134) and sialostatin L2 (135), both present in tick saliva of *Ixodes scapularis*, were shown to inhibit cathepsin L from the host and potentially immunosuppress the vertebrate response. By modulating the inhibitory activity of the host elastinolytic proteases cathepsins V, L and S, secreted from the host macrophages, ticks may preserve the feeding cavity intact and in this way facilitate blood feeding (135). Given the role of the targeted enzymes (136, 137) in vertebrate immunity, tick sialostatins L and L2 may interfere with cathepsins V, L and S, which are directly involved in antigen presentation inside APC (dendritic cells, macrophages). This means that these inhibitors could interfere with the modulation of controlled degradation of MHC II associated chaperone invariant chain (Ii) to CLIP or to interfere with the degradation of antigen to antigenic peptides, both inside the MHC II loading compartments. Host immunomodulation has been implicated in the deleterious phenotype of ticks, silenced for sialostatin L2, which further makes *I. scapularis* cystatins attractive targets for development of anti-tick vaccines (135).

Another example from the lone star tick *Amblyomma americanum* showed that disrupting the expression of salivary cystatin, by RNAi-mediated gene silencing, reduced the ability of ticks to feed successfully (138). Other roles have also been described. Cystatin HIcyst-2 from the gut of tick *Haemaphysalis longicornis* has been associated with tick innate immunity (139). Furthermore, two secreted type 2 cystatins, Om-cystatin 1 and Om-cystatin 2, from the soft tick *Ornithodoros moubata,* were described and suggested to play a role in

tick midgut physiology, rather than in salivary glands (140). A physiologically relevant role still has to be elucidated for stefin type inhibitor Bmcystatin from tick *Boophilus microplus* (141). It may be associated with regulation of proteolysis during embryogenesis or with regulation of the host immune system, as speculated for Bmcystatin-like protein found in tick salivary gland (141).

7. PERSPECTIVE

Many cystatins show differential expression and/or localization, as well as changes in secretion, on different stimuli acting on immune cells. Understanding the regulatory roles of cystatin inhibitors towards the proteolytic enzymes present in immune and other cells associated with the immune system, will provide us with valuable information as to how an immune response could be modulated in order to treat a specific disease. Cystatins from parasites have multiple, but specific, capacities for immunomodulation acting in parallel on different immune effector mechanisms. Therefore, much can be gained from understanding pathogenic organisms and parasites that use their own cystatin inhibitors to modulate proteolytic processes within the host cells and in this way to interfere with their immune response. The elucidation of these mechanisms could be crucial for the development of novel immunotherapeutic agents.

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Abbreviations: Ag: antigen, APC: antigen presenting cells, MHC II: major histocompatibility complex class II molecules, TGF-beta: transforming growth factor beta, TNF-alpha: tumor necrosis factor alpha, IFN-gamma: interferon gamma

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