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Helminth-derived cystatins: the immunomodulatory properties of an *Ascaris lumbricoides* cystatin

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Review

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Abstract

Helminth infections such as ascariasis elicit a type 2 immune response resembling that involved in allergic inflammation, but differing to allergy, they are also accompanied with strong immunomodulation. This has stimulated an increasing number of investigations, not only to better understand the mechanisms of allergy and helminth immunity but to find parasite-derived anti-inflammatory products that could improve the current treatments of chronic non-communicable inflammatory diseases such as asthma. A great number of helminth-derived immunomodulators have been discovered and some of them extensively analysed, showing their potential use as anti-inflammatory drugs in clinical settings. Since *Ascaris lumbricoides* is one of the most successful parasites, several groups have focused on the immunomodulatory properties of this helminth. As a result, several excretory/secretory components and purified molecules have been analysed, revealing interesting anti-inflammatory activities potentially useful as therapeutic tools. One of these molecules is *A. lumbricoides* cystatin, whose genomic, cellular, molecular, and immunomodulatory properties are described in this review.

Introduction

The prevalence of inflammatory non-communicable diseases (IND), for example, inflammatory bowel disease, diabetes, obesity and asthma, is increasing (Lin *et al.*, 2020; Zwicky *et al.*, 2020), and there are reasons to believe that the control of infectious diseases, including those caused by helminth parasites, influences this epidemiological trend (Bach, 2002; Platts-Mills, 2015; Caraballo, 2018). Although important advances have been made on asthma, especially in detecting risk factors and pathophysiological mechanisms initiated by allergens and environmental pollutants, it can be said that improvements in terms of treatment and prevention are needed. In fact, the prevalence of asthma has increased over the past 30 years in almost all countries where it has been investigated, and treatment has not progressed substantially in the past 15 years (Bush *et al.*, 2015). The advent of biologics, such as monoclonal anti-cytokine antibodies, is increasing, but these products are indicated only for patients with severe asthma, who are no more than 15% of asthmatics (O'Toole *et al.*, 2016; Hossny *et al.*, 2017).

One of the advances on asthma pathogenesis is the increasing understanding of the impairment of the regulatory mechanisms associated with the disease (Roth-Walter *et al.*, 2020), which could lead to an overexpression of the type 2 immune response against allergens. Besides, basic and clinical investigations have revealed that helminth infections induce modulation (mainly associated with suppression) of the immune responses. In fact, even though the allergic response and helminth immunity share several characteristics, one of the main differences between them is the role of immunomodulation, which is low in allergy and high during helminthiases. This immunomodulation is elicited by numerous parasite-derived molecules, whose significant anti-inflammatory effects have opened a new hope for IND treatments.

The most frequent soil-transmitted helminth infection is ascariasis, caused by *A. lumbricoides* (Pullan *et al.*, 2014). In highly endemic zones, this disease is thought to produce a state of general immune hyporesponsiveness that impedes the efficacy of viral and bacterial vaccines (Labeaud *et al.*, 2009; Salgame *et al.*, 2013). But more recently, it has been shown that, regarding allergy, ascariasis can exert a dual effect on the host. Heavy intensity infections are associated with a reduction of allergic symptoms, while low parasite burden infections are related to increased allergy symptoms (Caraballo, 2018; Zakzuk *et al.*, 2018). The causes of these two general outcomes are unknown but probably have to do with the level of exposure and host genetic susceptibility (Caraballo, 2018). However, the mechanisms of *Ascaris*-induced immunosuppression have been investigated during the last years and several anti-inflammatory products have been isolated and tested, among them the cysteine protease inhibitors, also known as cystatins. In this review, we describe the evolution and findings of the experimental studies on the immunomodulatory properties of helminth-derived cystatins, with special focus on the *A. lumbricoides* cysteine protease inhibitor (Al-CPI) and its effects on a model of allergic airway inflammation.

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The role of immunomodulation in host–parasite relationships

General mechanisms of immunity to A. lumbricoides

The relationships between Ascariasis and asthma have been studied in view of the similarities and differences between the immune mechanisms of both conditions and to better understand allergy and *Ascaris* immunity. However, the information about the natural immune response to *Ascaris lumbricoides* is very scarce and most of the analyses are the sum of experimental studies with various helminths, several of them infecting non-natural hosts. For human infection with *A. lumbricoides* (ascariasis), it has been exceedingly difficult (for various reasons including ethical ones) to define the defence mechanisms, so the information is limited; as a result, it is one of the infections with less coherent information, despite its high prevalence and disease burden. Here we make a brief comment on studies evaluating the immune responses in humans and pigs, an experimental model where natural infection with *Ascaris suum* resembles ascariasis in humans.

Intestine

According to the life cycle of *A. lumbricoides*, the intestine is its first meeting place with the defence system. Research on the immune response at this level is scarce and has focused on antibody production, the cellular response and the ability to eliminate the parasite (Geiger *et al.*, 2002; King *et al.*, 2005). To overcome the limitations of human experimentation, studies have been conducted in pigs infected with *A. suum*. In these animals, most larvae are eliminated from the intestine between 14 and 21 days (Masure *et al.*, 2013*b*). Several investigators have identified mechanisms that might be relevant to humans with ascariasis and the factors that influence the progression or control of the infection (Jungersen *et al.*, 1999; Masure *et al.*, 2013*b*). In these and other studies, the role of eosinophils has been investigated using different approaches; for example, *in vitro* degranulation of eosinophils was elicited by L3 larvae in the presence of serum from pigs immunized with infective *A. suum* eggs and then challenged with L3 larvae. Interestingly, this effect was abolished when inactivating the serum, suggesting the participation of the complement system and the action of eosinophils in early larval stages (Masure *et al.*, 2013*a*).

Other studies have attempted to clarify the role of antibodies, revealing a local production of cells secreting IgM and IgA antibodies. IgA is secreted by cells residing in the lamina propria of the proximal jejunum. The highest IgA levels coincided with the larval elimination period (Miquel *et al.*, 2005). Although these immune mechanisms may theoretically lead to total larvae expulsion, sterile immunity is not frequently observed. Elimination of infective eggs and L3 larvae has been found in challenged pigs (Masure *et al.*, 2013*b*). Microarray analysis reveals a differential gene program in L4 larvae which makes them more suitable to parasitize the host and evade the immune response (Morimoto *et al.*, 2003). In summary, the intestine is the main local response site against *Ascaris* spp., and although the mechanisms are not yet well defined, some experiments suggest that, in addition to the cellular response, especially by eosinophils and intraepithelial T lymphocytes, antibodies could be useful by preventing migration of the parasite and supporting its elimination.

Liver

Most studies evaluating immunity in the liver have been carried out in mice. In humans and pigs, a local immune response has been observed, which does not prevent the migration of the parasite to other organs such as the lung; however, some strains of mice manage to do so, although the mechanisms are unclear (Lewis *et al.*, 2006; Dold *et al.*, 2010; Deslyper *et al.*, 2016). The existence of genetic variants in humans controlling the larval load at different stages of the infection cycle is possible, but this needs to be demonstrated. In studies carried out in pigs, factors other than genetics have been evidenced that could influence the blocking of *A. suum* migration and the complexity of the lesions at the liver level (Eriksen *et al.*, 1980; Serrano *et al.*, 2001); however, there is no satisfactory explanation as to why *Ascaris* is not eliminated in the liver and continues its path to the lung.

Lungs

There is no clear evidence showing a protective effect of the immune response against *Ascaris* spp. at the pulmonary level; however, as it passes through the lungs, an intense local response has been evidenced that can induce respiratory symptoms such as cough and dyspnoea, and some severe inflammation like in Loeffler's syndrome (Holland *et al.*, 2013). These symptoms are the product of an inflammatory response induced either by the presence of the larvae and its products locally, remotely or both. Doctors from endemic areas observe that parasitized children present occasional respiratory symptoms, which are supposed to be induced by the migration of the larvae; however, there are no studies that objectively demonstrate this condition, although some have indirectly explored it (Lynch *et al.*, 1992; Lewis *et al.*, 2006) To evaluate the immune response at this level, studies have been designed in both mice and pigs. They have described the local changes that occur with the passage of *A. suum* through the lungs, suggesting that, of the cell types found, eosinophils play a fundamental role in the elimination of parasites (Dawson *et al.*, 2005; Enobe *et al.*, 2006).

Systemic response

During their entry through the intestinal mucosa and their migration to the liver and lungs, in addition to the local inflammatory response, *Ascaris* larvae induce immunological changes that can be detected in blood and tissues. These changes include cell mobilization, the production of specific antibodies, the activation of various genes and the production of a wide variety of proteins such as cytokines and chemokines. One of the best known systemic immune changes of ascariasis is the increase of total IgE levels, which is derived, at least in part, from a polyclonal stimulation of B lymphocytes by products of the parasite's body fluid (Lee and Xie, 1995). Eosinophilia is also widely known (Jungersen *et al.*, 1999; Medeiros *et al.*, 2006). There is antibody production both in animals (Lejkina, 1965) and humans; almost all isotypes and also subclasses of IgG have been detected, although their role in immunity against the parasite is still debated (King *et al.*, 2005) because there is only epidemiological evidence (McSharry *et al.*, 1999; Turner *et al.*, 2005), although, as mentioned, the presence of the immune serum was essential to kill the larvae (Masure *et al.*, 2013*a*). There is evidence that IgA, both local and serum, could be protective (Miquel *et al.*, 2005). Antibodies are directed against a wide variety of protein antigens detected by WB extracts or purified molecules and also against glycolipids (van Riet *et al.*, 2006).

Cytokine measurement suggests that in children with ascariasis, there is a strong polarization towards a Th2 profile measured by stimulation with various larval stages of the parasite (Cooper *et al.*, 2000). This response appears to increase after repeated treatment with albendazole (Cooper *et al.*, 2008), which could be the result of low parasite burden reinfections that reverse the immunomodulation induced by chronic infections. *In utero* sensitization against *Ascaris*-specific antigens has been observed studying the cellular response of the umbilical cord in children born to infected mothers (Guadalupe *et al.*, 2009). An interesting aspect of the immune response against *Ascaris*, as with other

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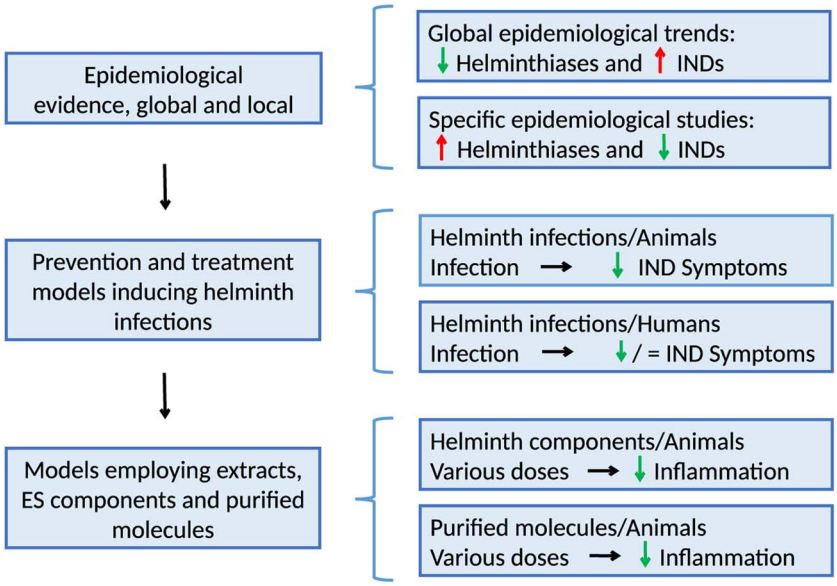

Fig. 1. The evolution of research on helminth immunomodulation includes epidemiological observations at the local and general levels, case–control studies, treatment trials using helminths, mechanisms of action in animal experimentation and *ex vivo* assays. IND, inflammatory non-communicable disease.

helminths, is its ability to completely eliminate parasites and establish permanent immunity (Bourke *et al.*, 2011). The complete elimination of parasites in natural hosts is not commonly seen, probably because a protective and immunomodulatory response is established that does not eradicate the parasite but limits its reproduction and damaging effects (Zakzuk *et al.*, 2018). According to genetic considerations, it is theoretically possible for one group of the population to undergo a self-cure process and another group to be intensely infected (Caraballo, 2013).

Evolution of parasitic immunomodulation studies

The immunomodulation by parasites was first proposed by Greenwood to explain the low prevalence of autoimmune diseases in Nigeria (Greenwood, 1968) and then by Gerrard *et al.* to explain the higher frequency of asthma, eczema and urticaria in the white compared to the Metis populations in Canada (Gerrard *et al.*, 1976). These observations have been followed by a great number of epidemiological studies demonstrating the beneficial effects of different helminthiases on IND, for example, the inflammatory bowel disease and asthma (Maizels, 2016). Also, the possibility that the increasing trends of the prevalence of allergic diseases are related to the parasitic infection control has been considered (Platts-Mills, 2015; Caraballo, 2018). At the experimental level, several animal models employing a variety of compounds, sometimes purified, have been used (Coronado *et al.*, 2017). More recently, *ex vivo* experiments have been particularly useful for analysing the cellular and molecular effects of recombinant immunomodulators (Coronado *et al.*, 2019). The evolution of research on helminth-derived immunomodulators is summarized in Fig. 1.

General mechanisms of helminth immunomodulation

Helminth infections induce species-specific and other more generalized immune responses that may be critical for disease control, but also induce immunomodulation. Two of the most important immunomodulatory effects are systemic activation of type-2 immune responses and immunosuppression. Advances in helminth genomics, proteomics and other systematic biology disciplines have uncovered a wide number of molecules with the potential to modulate host defence and bystander immune responses. Besides proteins, the repertoire of immunomodulatory products derived from helminth includes lipids (Giera *et al.*, 2018) and glycans (Maizels *et al.*, 2018) that activate innate immune responses and play a significant role in immunomodulation. Also, the list of allergenic proteins is growing, and different animal models have shown that they could exert tissue damage through type-2 immune mechanisms and innate immune pathways. The vast number of mechanisms which are relevant in helminth innate immune responses include complement system (Giacomin *et al.*, 2008), mucosal barrier (Gerbe *et al.*, 2016), neuroimmune networks (Cardoso *et al.*, 2017), ILC2 (Herbert *et al.*, 2019) and more classically described granulocyte-mediated mechanisms (Masure *et al.*, 2013*a*). Also, the role of antigen-presenting cells on immunomodulation is well-studied, even though not well-defined. Induction of tolerogenic APCs has also been linked to helminth immunomodulation; these cells are characterized by predominant IL-10 production and surface expression of ligands of molecules expressed in T cells that may modulate its activity. Other mechanisms include sequestration of adaptor molecules leading to suppression of pivotal innate immune networks: omega-1 is a ribonuclease that targets the global pool of RNA and in this way suppress DC activation through inhibition of CD86 and MHCII expression (Everts *et al.*, 2012). By means of different models of helminth infections, it has been described that specialized innate cells drive type 2 immune polarization. In dendritic cells, parasite products may reduce co-stimulatory molecules and MHC expression which inhibit T-cell activation.

Molecular and genetic aspects of *A. lumbricoides* cystatins

Structure and biologic function of cystatins

Cystatins are a superfamily of cysteine protease inhibitors that commonly occur as single-domain proteins and mainly inhibit peptidases from the papain (C1) and legumain (C13) families (Turk and Bode, 1991). They were annotated to the gene ontology term 'cysteine-type endopeptidase inhibitor activity' (GO 0004869:); by definition: 'stops, prevents or reduces the activity of a cysteine-type endopeptidase, any enzyme that hydrolyses peptide bonds in polypeptides by a mechanism in which the sulfhydryl group of a cysteine residue at the active centre acts as a nucleophile' (geneontology.org). Cystatins bind tightly and reversibly to cysteine proteases inhibiting their activities, thus preventing, or controlling protein degradation (Rawlings *et al.*, 2004). They are ubiquitously found in most organisms and share a

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highly conserved motif of five amino acids (Gln-*x*-Val-*x*-Gly) in the central part of their sequences as well as an α helix cradled in a sheet of five antiparallel β strands in the tertiary structure (Kordis and Turk, 2009). Other secreted extracellular proteins such as kininogen, histidine-rich glycoproteins and fetuins also contain cystatin domains and play several functions in addition to inhibiting endogenous thiol proteinases (Turk *et al*., 2008; Shamsi and Bano, 2017).

The inhibitory mechanism of cystatins relies on the binding to the active site of a protease by a 'tripartite wedge'. This consists of a wedge-shaped group of mostly hydrophobic residues that lie at one edge of the β-sheet but arise from three regions on the protein sequence, namely the flexible N-terminus and the two loops between the β-strands. The conserved motif Q-*x*-V-*x*-G (where *x* is usually a small amino acid such as alanine or serine) is in a tight turn between β-strands two and three. The hydrogen-bond ladder between strands two and three extends all the way to the valine in the loop, which forms a hydrogen bond with the glycine. This holds the valine in an unusually strained conformation that enables cystatin to fit into the active site of cysteine proteases and may also help prevent the inhibitor from becoming a substrate of those proteases. Once these residues insert into the active-site groove of the protease, they block access to substrate proteins; the shape of this wedge is complementary to that of the groove, which contributes to the tight binding. Two cystatin molecules can also do 'domain swapping' resulting in higher stability or for some vertebrate cystatins allows their oligomerization (Amin *et al*., 2020).

Members of the cystatin superfamily are structurally and functionally related, and according to the Pfam annotation (PF00031, https://pfam.xfam.org/family/Cystatin), members of this family can be classified into four types that consider their structural and functional relationships, cellular location and polypeptide features, as follows:

Type 1 or stefins: These are mainly intracellular cystatins but may also appear in body fluids. They are single-chain polypeptides of about 100 residues, without disulphide bonds nor carbohydrate side chains, and with a molecular weight of about 11 kilodaltons (kDa). Stefins inhibit peptidases of the C1 family, for instance lysosomal cathepsins.

Type 2 cystatins: These are extracellular secreted polypeptides largely acidic and synthesized with a 19–28 residue signal peptide. They are about 120 amino acids long, contain conserved cysteine residues that form disulphide bonds and can be glycosylated or phosphorylated. Type 2 cystatins are broadly distributed and found in most body fluids where they inhibit proteases found in the extracellular space, mainly of the C1 family and legumain.

Type 3 or kinin precursor proteins (kininogens): These proteins contain multiple cystatin domains in the same polypeptide chain. In mammals, there are three different types of kininogens: H- (high molecular mass), L- (low molecular mass) kininogen and T-kininogen that is only found in rat. Kininogens ubiquitously exist in vertebrates, including mammals, birds, amphibians and fishes. They vary extremely in both structure and function among different taxa of animals and typically contain a bradykinin domain and one cystatin domain in lampreys, two cystatin domains in fishes or three in mammals, birds and amphibians (Lee *et al*., 2009). Kininogens can inhibit calpain and peptidases in families S8 and M12.

Unclassified cystatins: Also designated cystatin-like proteins, this family includes secreted cystatin-like proteins without inhibitory activity such as fetuins and histidine-rich proteins, restricted to vertebrates and phytocystatins from plants. Fetuins can be classified as fetuin-A and fetuin-B and are characterized by the presence of two N-terminally located cystatin-like repeats and a unique C-terminal domain that is not present in other proteins of the cystatin family.

A more recent classification of cystatins has been proposed in the MEROPS database (Rawlings and Bateman, 2021) naming cystatins with the letter 'I' for inhibitor and the number 25 (I25), followed by 'A' for type 1 stefins (I25A), 'B' for type 2 cystatins (I25B) and 'C' for type 3 kininogens (I25C). For more information, visit (https://www.ebi.ac.uk/merops/cgi-bin/famsum?family=I25). Although these classifications are in most cases very useful, for some cystatins can be difficult to apply because there are combinations of different features that may not be attributed to one or other family (Ilgova *et al*., 2017), or multidomain cystatins that albeit of high molecular weight (170 kDa), tandem cystatin repeats and degenerated core motifs still resemble type 2 cystatins (Geadkaew *et al*., 2014).

Genomics of cystatins

The MEROPS database (Rawlings *et al*., 2014) reports at least 4301 cystatin sequences (https://www.ebi.ac.uk/merops/inhibitors/) and the SMART database reports at least 9885 cystatin-like domains in 7379 proteins (https://www.ebi.ac.uk/interpro/entry/smart/SM00043/). The taxonomic distribution of proteins containing cystatin-like domain (s) is presented in Fig. 2a. Despite their structural diversity, most can inhibit proteinases indicating the conservation of this function. Most cystatins are found in metazoan (76.5%) and viridiplantae (21%) (Fig. 2a). Phylogenomic analysis of the cystatin superfamily tracked their origin prior to the divergence of the major eukaryotic lineages. The progenitor of this superfamily was most probably intracellular and lacked a signal peptide and disulphide bridges. Cystatin from *Giardia* resembles the most ancestral eukaryotic cystatin (Kordis and Turk, 2009). A primordial gene duplication produced two ancestral eukaryotic lineages, stefins and cystatins (Muller-Esterl *et al*., 1985). Stefins (Pfam:PF00031) remain encoded by a single or a small number of genes in eukaryotes, whereas cystatins have undergone a more complex and dynamic evolution through numerous gene and domain duplications. In some multicellular eukaryotes, three major bursts of functional diversification have occurred: one in land plants (angiosperms), the second during the evolution of the vertebrates and a third in the ancestor of placental mammals (Margis *et al*., 1998). In plants, cystatins formed a distinct branch called phytocystatins. In vertebrates, there are at least 4360 cystatin-domain-containing proteins with a mutation rate that is faster than in other groups of proteins (Rawlings and Barrett, 1990). Some taxonomic groups such as Fungi, Kinetoplastida and Apicomplexa have lost stefins and cystatins (Kordis and Turk, 2009).

After the analysis of several genomes, it has been established that within metazoan, the highest frequency of cystatins is found in the phylum Chordata (60%), followed by the phyla Arthropoda (12%), Nematoda (3.43%) and Platyhelminthes (0.5%) (Fig. 2a). It seems that the frequency of cystatins in the last three phyla could relate to promoting parasitism and immunomodulation. Indeed, several cystatins have been characterized in parasitic organisms (Klotz *et al*., 2011*a*). Cystatins in the salivary glands of ticks are thought to help bypassing the immune response, especially in hard ticks that require to attach to a host for long-term feeding; examples of these arthropod cystatins include syalocystatins, iristatin and Hisc-1 from ixodid ticks (Martins *et al*., 2020). Moreover, transcriptional analysis in *Strongyloides* ssp. have found that genes encoding cystatins are relevant for parasitism since they are upregulated in parasitic females compared with free-living females (Hunt *et al*., 2017).

There are at least 1161 genes coding for cystatin-domain-containing proteins in the phylum Nematoda annotated in the genomes from WormBase Parasite (v15.0). In parasitic nematodes, most characterized cystatins are type 2, although there

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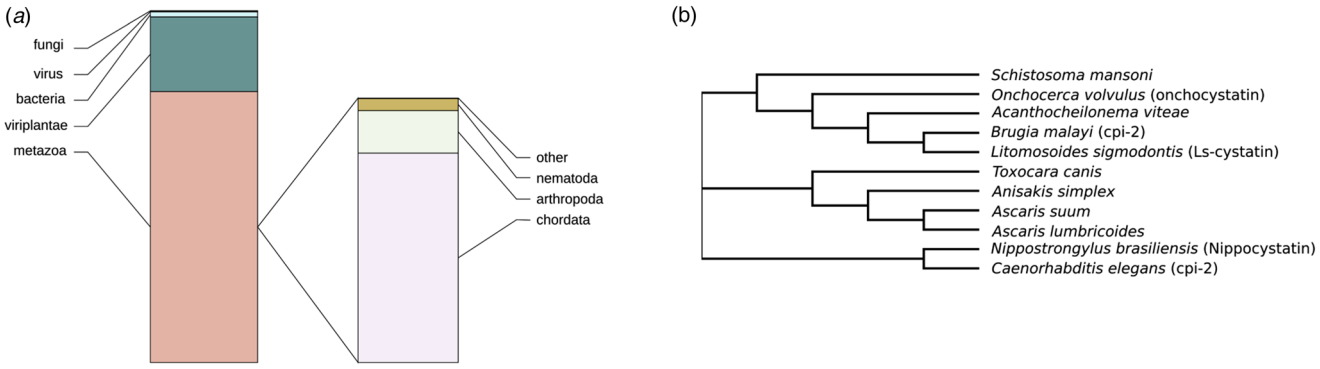

Fig. 2. (a) Taxonomic distribution of cystatin-domain containing proteins based on the SMART database (SM00043, accessed November 2020), the size on the stacked bar represents the percentage of proteins with the cystatin domain in each taxonomic node. (b) Cladogram of a phylogenetic neighbour-joining tree without distance corrections based on the sequences of type 2 cystatins in parasitic nematodes.

are also type 1, and others with multidomains. Nematode type 2 cystatins are found in parasitic organisms, including onchocystatin from *Onchocerca volvulus*, Av-cystatin from *Acantocheilonema vitae*, Bm-CPI-1 and Bm-CPI-2 from *Brugia malayi*, Al-CPI from *A. lumbricoides* and nippocystatin from *Nippostrongilus brasiliensis*. They are also found in free-living organisms such as *Caenorhabditis elegans* (Fig. 2b). Cystatins regulate physiological processes such as larval moulting and play a critical role as immunomodulators in host–parasite interactions by inhibiting cathepsins, antigen processing and presentation, the expression of pattern recognition receptors and modulating cytokines and nitric oxide production (Khatri *et al.*, 2020). Some of these immunomodulatory properties have been also described for cystatins in the Platyhelminthes *Fasciola hepatica* and *Schistosoma* spp.; although comparative analysis revealed particular structural features in cystatins from cestodes (Guo, 2015). In the following section, we describe the biological features of cystatins in the human parasitic nematode *A. lumbricoides*.

Ascaris lumbricoides *cystatins*

The MEROPS database reports at least 243 protease inhibitors (known and/or putative) in *A. lumbricoides* including four cystatins (family IH, clan I25). They are encoded by the genes ALUE_0000175801, ALUE_0002323401, ALUE_0001430001 and ALUE_0001868501 and predicted to be translated in the *A. lumbricoides* proteome to cystatins of 69, 107, 131 and 138 amino acids, respectively (Fig. 3a.) (UP000036681, TrEMBL, https://www.uniprot.org/proteomes/UP000036681). The 69 amino acids cystatin was found to be encoded by a transcript of 237 bp and a single exon; while the 107 amino acids cystatin is encoded by a transcript of 351 bp in three exons, both having 13 homologues in the Onchocercidae family. On the other hand, the 138 amino acids cystatin is encoded by a transcript of 417 bp and four exons, with 169 orthologues within several nematode families including cpi-2 from *C. elegans*. These transcripts suggest that *A. lumbricoides* has at least four cystatins, with and without signal peptide, with different lengths and amino acids substitutions (Fig. 3a).

An experimentally verified cystatin of *A. lumbricoides*, denominated *cpi* or Al-CPI, is encoded in the forward strand of the ALUE_0001430001 gene (WormBase https://parasite.wormbase.org) and spans in four coding exons transcribed to 523 base pairs (bp) (ALUE_0001430001-mRNA-1) (Fig. 3b). This gene is translated to the *A. lumbricoides* cystatin (E9N3T6), which has 131 amino acids and a molecular weight of 14.2 kDa. It has a signal peptide (a.a. 1–20) and a cystatin domain between amino acids 23 and 131 (Fig. 3b). This cystatin also has two disulphide bonds (a.a. 88–98) and (a.a. 109–129) and the biochemical features of a type 2 cystatin. Mei *et al.* determined the crystal structure of this cystatin to 2.1 Å resolution (PDB 4it7) (Mei *et al.*, 2014) and demonstrated that Al-CPI strongly inhibited the activities of cathepsin L, C, S and, in a lower level, of on cathepsin B. Two segments of Al-CPI loops 1 and 2 were proposed as the key structural motifs responsible for Al-CPI binding to proteases and its inhibitory activity. Comparative sequence analysis revealed that Al-CPI has 29 orthologues including two homologues in the pig roundworm (92% identity with the cystatin from *A. suum*: A. suum: F1LHQ3), two homologues in *Parascaris* ssp. (85% identity with the cystatin of *Parascaris univalens*) and 71.5% identity with the cystatin of *Toxocara canis* (Fig. 4b). Interestingly, the amino acid identity of Al-CPI with the cystatin Ani s 4 from the fish-infecting nematode *Anisakis simplex* and type 2 cystatins in free-living species such as *Caenorhabditis* ssp., drops to 40%. Al-CPI also has sequence homology ranging from 48 to 33% with the other three sequences annotated as cystatin-domain-containing proteins in the *A. lumbricoides* genome (Fig. 5a).

Data on expression profiles of *A. lumbricoides* cystatins at the transcriptional or protein levels are extremely limited. However, at least five homologous cystatins are annotated in the *A. suum* genome with available RNAseq data in different tissues and lifecycle stages (Jex *et al.*, 2011; Wang *et al.*, 2011; Rosa *et al.*, 2014). We extracted the data on cystatin levels and the results suggest remarkable differences in cystatin mRNA expression between different *A. suum* tissues (e.g. embryo *vs* testis) or stages (Fig. 5b). In addition, a proteomic analysis of *A. suum* fluid compartments and secretory products confirmed the presence of CPI-2a cystatin-like inhibitors in the perienteric and uterine fluids of the adult worm (Chehayeb *et al.*, 2014). Cystatins have been also detected in extracellular vesicles and secretion/excretion body fluids from *A. suum* (Hansen *et al.*, 2019). Detailed transcriptome and proteomic analyses of *A. lumbricoides* are needed to verify these results and the relationship between cystatin expression during the different lifecycle stages, as well as specific effects of cystatin variants during host–parasite interactions.

Ascaris lumbricoides-induced immunomodulation

General aspects of the immunomodulation induced by A. lumbricoides

The mechanisms of helminth immunomodulation vary with the species, and some of them have been extensively studied (Homan and Bremel, 2018; Zakeri *et al.*, 2018; de Ruiter *et al.*, 2020; Wiedemann and Voehringer, 2020); however, basic research

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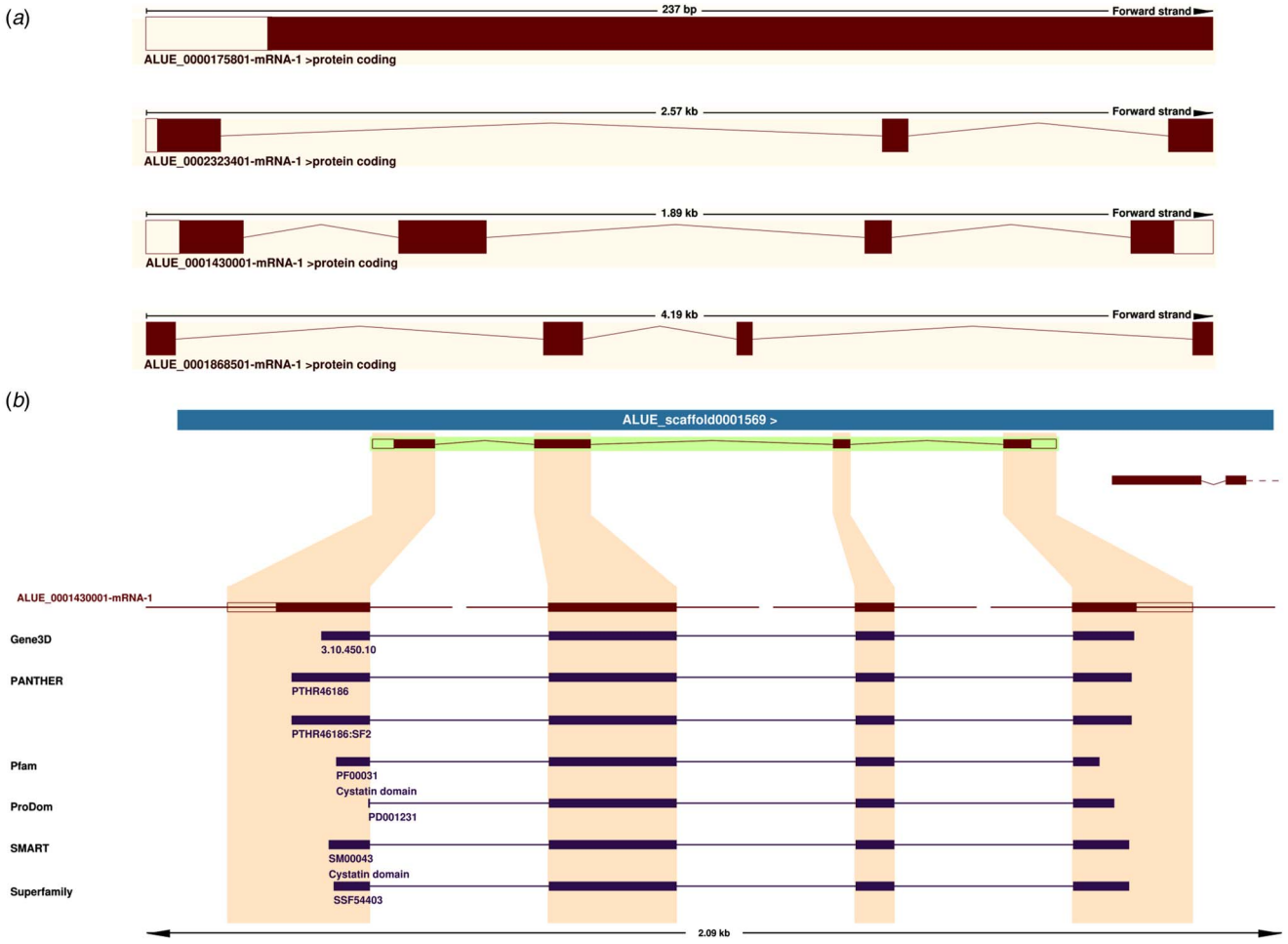

Fig. 3. (a) Transcript structure of four cystatins in *A. lumbricoides* (genome PRJEB4950, assembly Ecuador_v1_5_4) encoding the 69 amino acids (aa), 107 aa, 131 aa (Al-CPI) and 138 aa, respectively. (b) Annotation of the protein domains and features in the transcript ALUE_0001430001-mRNA-1 encoding the experimentally verified *A. lumbricoides* cystatin (Al-CPI).

on the mechanisms of *Ascaris*-induced immunomodulation is limited. *Ascaris* spp. are able to downregulate the immune responses of hosts (Barriga, 1984; Faquim-Mauro and Macedo, 1998; Caraballo *et al.*, 2019), which explains why they are among the most successful parasites. This has been shown in humans and experimental animals, using various strategies. For example, different patterns of cytokine production have been detected among young adults living in an *A. lumbricoides* endemic area. Chronically infected *Ascaris* and *Trichuris* patients with high parasite load presented reduced PBMC reactivity and lower type 1 cytokines TNF-α, IFN-γ and IL-12, than those non-infected endemic controls (Geiger *et al.*, 2002). This immune hypo-responsiveness has been associated with greater frequencies of the spontaneous production of IL-10 (Figueiredo *et al.*, 2010) and modified Th2-like immune phenotype (Reina Ortiz *et al.*, 2011) that can impair the immune response to co-infecting parasites (Hagel *et al.*, 2011) and the normal response to vaccines (Cooper *et al.*, 2001).

Whether *Ascaris*-induced immune hyporesponsiveness has an impact on preventing asthma or other allergic diseases remains to be defined, but recent reports show that soil-transmitted helminthiases during childhood protect from wheezing and asthma but not from allergen sensitization (Cooper *et al.*, 2017; Hamid *et al.*, 2017). Since *Ascaris* and other helminths co-infection is very frequent, it is difficult to evaluate their individual downregulatory properties in humans; however, progress on the molecular characterization of parasite immunomodulatory components is expected to facilitate this research.

The mechanisms of the immune downregulatory effects of *A. suum* infection have been recently explored in a mouse model of LPS-induced inflammatory response (Titz *et al.*, 2017), finding that infection suppressed secretion of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) and induces high levels of IL-10 and TGF-β, as well as CD4 + CD25high, Foxp3 + T cells in the mesenteric lymph nodes. This has also been investigated in pigs using transcriptomic analysis, showing that chronic *A. suum* infection induces suppression of inflammatory pathways in the intestinal mucosa, downregulating genes encoding cytokines and antigen-processing and co-stimulatory molecules. This effect was reproduced by *A. suum* body fluid in human dendritic cells *in vitro* (Midttun *et al.*, 2018).

The immunomodulatory capacity of *A. suum* and *A. lumbricoides* components has also been studied in animal models (Rocha *et al.*, 2008; Dowling *et al.*, 2011; Almeida *et al.*, 2018) and some immunomodulatory components found in excretory/secretory products of Ascaris have been identified. Among them, phosphorylcholine-containing glycosphingolipids (Deehan *et al.*, 2002; Kean *et al.*, 2006) and PAS-1 (Itami *et al.*, 2005; Antunes *et al.*, 2015) have been more analysed; however, according to the *A. suum* genome (Jex *et al.*, 2011), this genus might have more than 15 potential immunomodulators. In this regard, the recent analysis of the anti-inflammatory properties of *A.*

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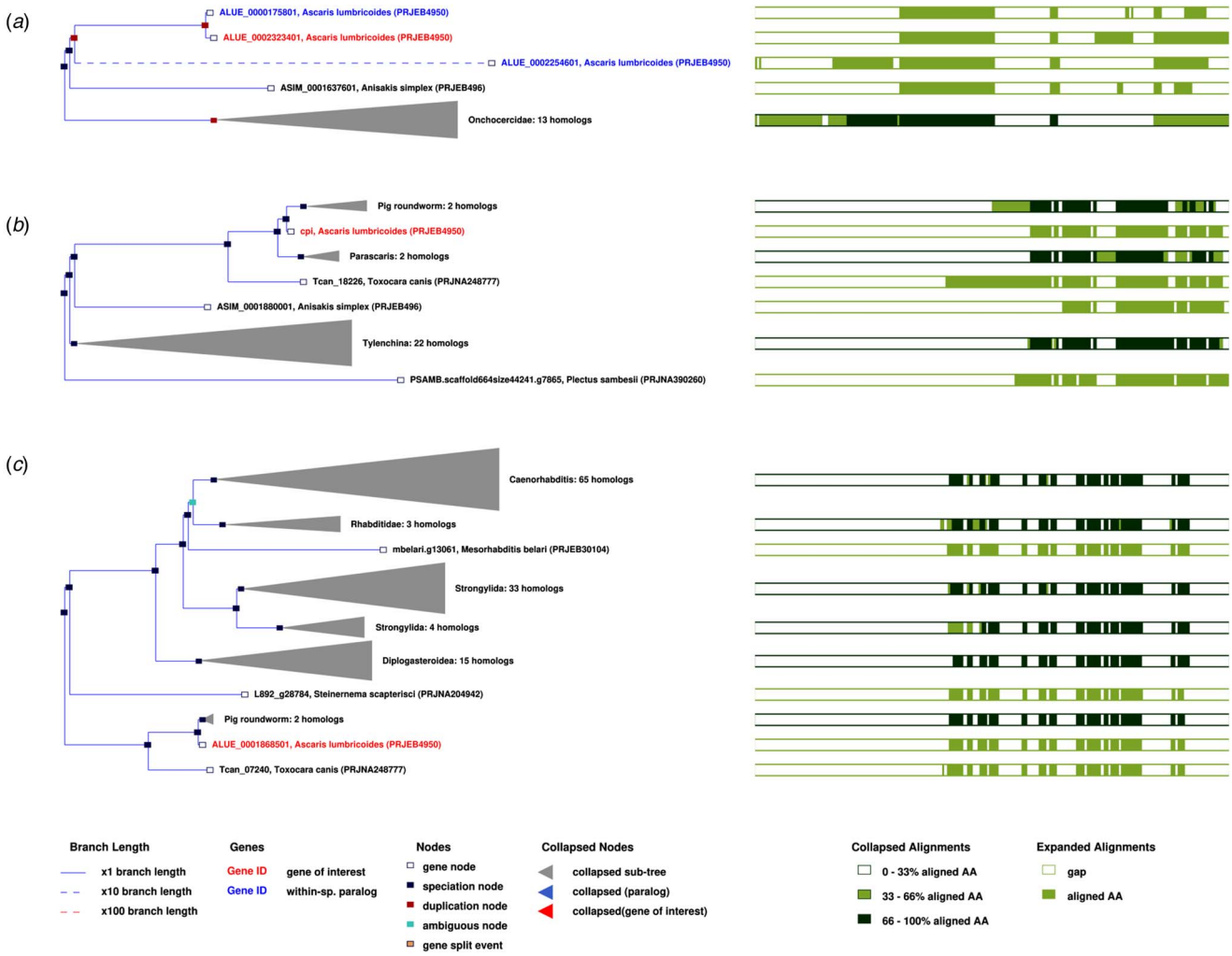

Fig. 4. Gene tree views of the four *A. lumbricoides* cystatins. (a) The 69 aa (ALUE_0000175801) and 107 aa (ALUE_0002323401) cystatins. (b) The experimentally validated *A. lumbricoides* cystatin (Al-CPI). (c) The 138 aa cystatin (ALUE_0001868501) which is the orthologue of cpi-2 from the free-living nematode *C. elegans*.

lumbricoides cystatin (Al-CPI) (Coronado *et al.*, 2017) started a new stage of research on the immunosuppressive potential of this nematode.

Immunomodulation by A. lumbricoides *cystatin*

Helminth cystatins are secreted during different developmental stages and participate in various physiological processes. As mentioned, they are classified into three classes, in which type-2 members are the ones linked to immunosuppression in mammals. According to the characterization of diverse cystatins from parasites, most common mechanisms of action include inhibition of MHC-II expression and antigenic presentation, an increase of nitric oxide production and the induction of regulatory cytokines (IL-10 and TGF-β) as well as a regulatory profile in macrophages and T cells. T-cell immunosuppression has also been described, but it is still an open question if cystatins act directly or it is mediated by innate immune cells (Hartmann *et al.*, 1997; Schonemeyer *et al.*, 2001).

Production of a recombinant Al-CPI

Genome sequencing of *A. suum* led to identify a sequence with homology to other helminth cystatins. Based on this, we designed primers to amplify this sequence from a cDNA library of *A. lumbricoides* and the amplicon was cloned directly to the pQE30 vector. This led to the production of *E. coli* of a His-tagged protein with the functional activity of cysteine protease inhibition. This recombinant protein was injected in mice at different doses to test its toxicity, observing it is a safe product that does not induce abnormal physical or behavioural patterns in treated animals (Coronado *et al.*, 2017).

Intestinal anti-inflammatory properties

Al-CPI has proved to prevent inflammation and tissue damage in a murine model of dextran sodium sulphate-induced colitis and asthma, adding evidence on its anti-inflammatory effects on different contexts of exacerbated and pathologic immune responses. In the colitis model, recombinant Al-CPI (rAl-CPI) was intraperitoneal administered daily for 15 days before and during the acute phase of inflammation. It was found that rAl-CPI reduced disease severity and epithelial damage. Colon samples of rAl-CPI mice had lower destruction of intestinal crypts and of goblet cell depletion. Mice treated with rAl-CPI showed high expression of IL-10 and TGF-β in colonic tissue together with the reduction of IL-6 and TNF-α RNA and protein levels (Coronado *et al.*, 2017). Like Al-CPI, four other helminth cystatins with therapeutic potential on inflammatory bowel disease have been reported (Schnoeller *et al.*, 2008; Jang *et al.*, 2011; Wang *et al.*, 2016; Togre *et al.*, 2018),

Anti-inflammatory properties in an allergy respiratory model

The house dust mite *Blomia tropicalis* is abundant in the Tropics, where it is an important source of allergens (Caraballo *et al.*, 2020) and one of the main risk factors for asthma. In another

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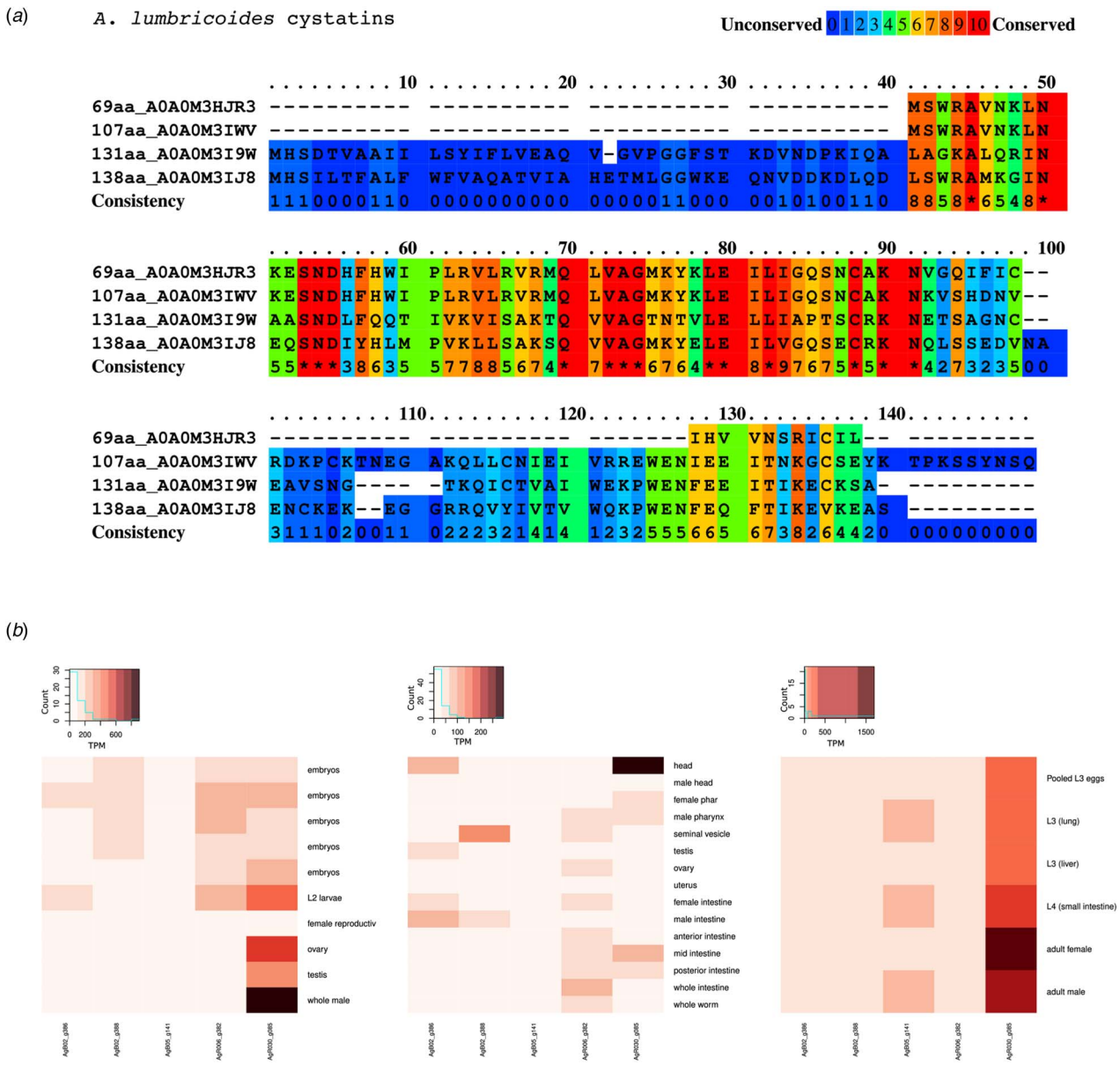

Fig. 5. (a) Multiple sequence alignment of four *A. lumbricoides* cystatins. (b) Gene expression profile of cystatins in *Ascaris suum* based on public RNAseq data from experiments SRP005511 (left), SRP013609 (middle) and SRP010159 (left) showing RNA expression levels in transcripts per million (TPK) in different tissues and lifecycle stages (data from public RNAseq studies downloaded from parasite.wormbase.org). The cystatin annotated as AgR030_g085 in *A. suum* is the equivalent of Al-CPI in *A. lumbricoides*.

study, we found that, similar to other helminth cystatins (Danilowicz-Luebert *et al.*, 2013; Ji *et al.*, 2015), Al-CPI reduces allergic airway inflammation, airway hyper-reactivity and other hallmarks of allergic inflammation after *B. tropicalis* sensitization, including eosinophil airways infiltration, goblet cell hyperplasia and elevated Th2 cytokine levels in bronchoalveolar lavage and IgE production. In this model, rAl-CPI was intra-peritoneal administered, 4 h before sensitization, successfully reducing allergic inflammation (Coronado *et al.*, 2019). This agreed with the observation that rAl-CPI induced an immunoregulatory response that included systemic IL-10 and TGF-β production as well as a strong IgG2 response that may dampen the allergenic effects of *B. tropicalis* (Coronado *et al.*, 2019). Administration of rAl-CPI and *B. tropicalis* extract caused a significant elevation of IFNγ together with immunoregulatory cytokines, as observed for other *Ascaris* immunomodulators (Araujo *et al.*, 2008). As Al-CPI, other helminth cystatins have been tested in models of allergic airway inflammation, showing similar results (Schnoeller *et al.*, 2008; Danilowicz-Luebert *et al.*, 2013; Ziegler *et al.*, 2015).

Mechanisms of AL-CPI immunomodulation

Based on different observations, macrophages/monocytes and dendritic cells are thought to be the main target of cystatins immunomodulation. Klotz *et al* found that filarial cystatin is mainly captured by these cell populations when injected intraperitoneally (Klotz *et al.*, 2011*b*). Also, monocyte depletion from peripheral blood mononuclear cells resulted in the abolition of the immunomodulatory effects of *O. volvulus* cystatin (Schonemeyer *et al.*, 2001). It has been experimentally confirmed that several cystatins, including Al-CPI, block different cathepsins which participate in proteasome and proteolytic digestion in the endosomal compartment. *Brugia malayi* cystatin interferes directly with antigen processing and presentation by inhibiting

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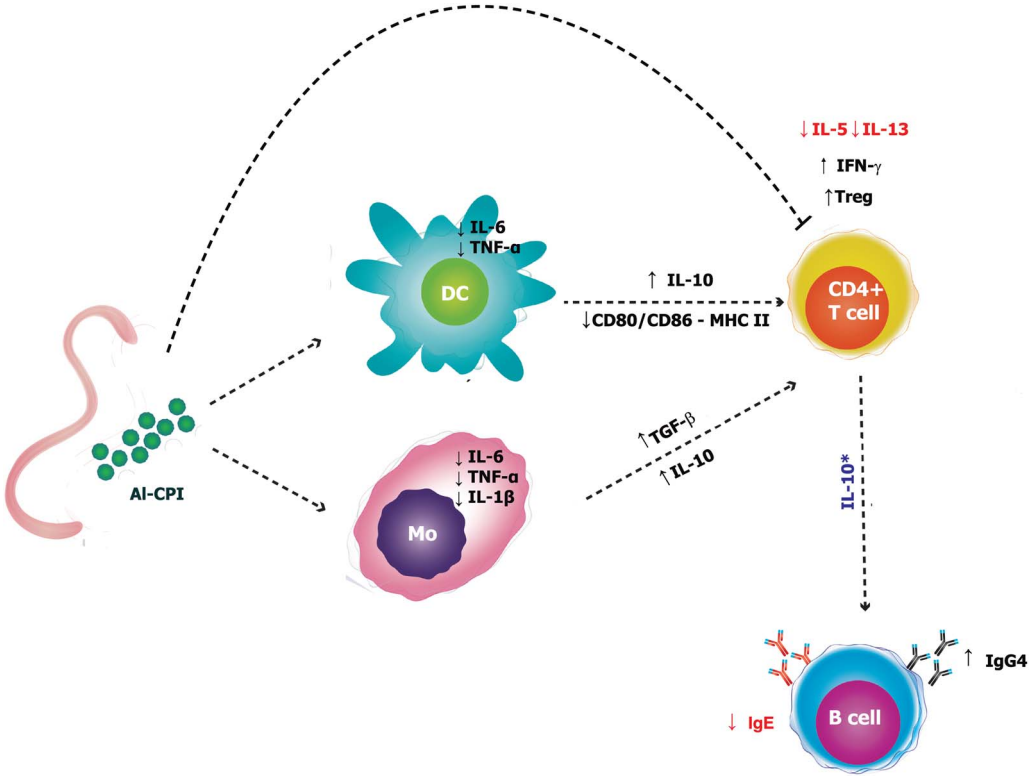

Fig. 6. Graphic integration of the different immunomodulatory effects of Al-CPI and the possible mechanisms of action leading to its immunomodulatory properties.

cysteine protease activity in endolysosomes (Manoury *et al.*, 2001; Murray *et al.*, 2005). Also, intraperitoneal treatment with recombinant *Nippostrongylus brasiliensis* cystatin (rNbCys) reduced 20% of the total activity of lysosomal cysteine proteases from the spleens or mesenteric lymph nodes. In this murine model, rNbCys reduced OVA-specific T-cell proliferation, probably due to its effects on antigen presentation (Dainichi *et al.*, 2001). Al-CPI strongly inhibit cathepsin L, C and S (Mei *et al.*, 2014), and it is possible that this enzymatic activity partially supports its immunomodulatory effects.

Inhibition of antigen processing results in an impairment of expressing MHC and co-stimulatory molecules on the cell surface in APCs (Kobpornchai *et al.*, 2020). Regarding Al-CPI, we found that it modulates the surface expression of co-stimulatory molecules (i.e. CD83 and CD86) in monocyte-derived human dendritic cells (Coronado *et al.*, 2019). For other cystatins, it has been demonstrated by co-culture experiments that cystatin-primed APCs induce lower T-cell proliferation (Manoury *et al.*, 2001; Sun *et al.*, 2013). Although not yet confirmed, our preliminary experiments showed that, in PBMC cultures, rAl-CPI significantly suppressed CD4 + CD3 + T-cell proliferation stimulated polyclonally with a reduction of IL-5 and an increase of IFN-γ levels, without changes in IL-10 production. Also, rAl-CPI reduced the *B. tropicalis*-induced production of IL-5 from allergic patients PBMC but not in healthy controls (Lozano *et al.*, 2020). We also found that Al-CPI may have direct effects on other cell populations because it inhibited the proliferation (about 60%) of purified CD3 + CD4 + T cells stimulated with CD-Mix, raising new questions about its mechanism of action (Lozano *et al.*, 2020). Hartmann *et al.* also found direct suppression of proliferation of purified CD3+ populations in murine T cells by AvCys (Hartmann *et al.*, 1997).

Besides its effects on antigen presentation, modulation of cytokine production by innate immune cells has been documented for different cystatins, both in human and mouse studies (Schonemeyer *et al.*, 2001; Yang *et al.*, 2014). Cystatins induce primarily a strong IL-10 production. In the case of rAl-CPI, *in vitro* stimulation of mouse peritoneal macrophages and spleen cells induced a parallel increase of IL-10, TGFβ, IL-6 and IFNγ. Similarly, the filarial cystatin increased IL-6 and IL-8 concomitantly with IL-10 (Venugopal *et al.*, 2017). As with other parasite cystatins, despite inducing this moderate production of inflammatory cytokines, the net effect observed with LPS is inhibition of its capacity to elicit IL-6, TNF-α and IL-1β secretion.

Although the inflammatory cytokines accompanying IL-10 production vary depending on the helminth cystatin or the cell population being analysed, this is an expected pattern of IL-10 secretion in innate immune cells. Several TLR ligands derived from bacteria, including LPS, induce IL-10 concomitant to the typical release of inflammatory cytokines (Saraiva and O'Garra, 2010). For filarial cystatins, it has been shown that the role of ERK, MAPK and p38-dependent pathways, which are known to participate in bacterial products, activated TLR signalling, on IL-10 induction (Klotz *et al.*, 2011*b*; Venugopal *et al.*, 2017). Therefore, it has been hypothesized that cystatins could have receptor-mediated effects, in addition to their inhibition of protease activity.

IL-10 was proposed to be a major mechanism of the protection from airway allergic inflammation induced by *Acanthocheilonema vitae* cystatin (Schnoeller *et al.*, 2008). When we assessed the relevance of this cytokine as a mechanism of action of Al-CPI to prevent airway allergic inflammation, we found that IL-10R blockade reduced Treg cell numbers and the local IL-10 production in the lung, while significantly increasing type 2 humoral responses. Despite these cellular effects, it did not fully counteract the effects of rAl-CPI on airway inflammation in BAL neither bronchial hyper-reactivity, suggesting that other mechanisms may be involved in rAl-CPI-mediated modulation of allergic responses induced by *B. tropicalis* (Coronado *et al.*, 2019). In agreement with this, Schönemeyer *et al.* observed that addition of

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anti-IL-10 antibodies did not restore the inhibition of anti-CD3-induced proliferation of PBMC by *O. volvulus* cystatin. This suggests that IL-10 is not a major component of the rOv17-induced inhibition of T-cell proliferation (Schonemeyer *et al.*, 2001). The role of other immunoregulatory mechanisms (TGF-β and Tregs) that are stimulated by cystatins, including rAl-CPI, deserves further investigation.

The increase of nitric oxide production (NO) has been proposed as another mechanism of action of cystatins. NO release has been associated with T-cell suppression, but it has also been reported that NO production by activated macrophages is associated with reduced parasite burden. rAl-CPI induced the expression of inducible nitric oxide synthetase (iNOS) in colonic tissue. Other cystatins, for example, those from *B. malayi*, *O. volvulus* and *A. vitae* (Hartmann *et al.*, 2002), induce NO secretion from primed macrophages. However, the relevance of these mechanisms on human macrophages remains to be investigated.

Concerns about the allergenic potential of cystatins have been raised by the recognition of the *Anisakis simplex* cystatin Ani s 4 as an allergen (Rodriguez-Mahillo *et al.*, 2007) as well as cystatins from other non-helminth allergenic sources (Ichikawa *et al.*, 2001). Also, native *A. vitae* cystatin – which is a strong immunosuppressive helminth product – elicits β-hexosaminidase release in rat basophil leukaemia cells sensitized with sera from immunized gerbils (Hartmann *et al.*, 2003), which suggest that it has allergenic activity. Although we could not definitely rule out this possibility, most of our results support that Al-CPI is poorly allergenic. First, bioinformatic analyses have shown that *A. lumbricoides* cystatins are homologous to other *Anisakis simplex* cystatins but not to Ani s 4. In addition, the nasal challenge with rAl-CPI did not induce an eosinophilic inflammatory response in airways, neither increased Penh values as observed with the *B. tropicalis* extract. Furthermore, rAl-CPI-specific IgE levels were undetectable in *B. tropicalis* sensitized/challenged mice perhaps justifying the failure of rAl-CPI to increase airway reactivity (supported by the Penh values) and induce passive cutaneous anaphylaxis (Coronado *et al.*, 2015).

In summary, Al-CPI appears to be a safe product with tolerogenic effects on antigen-presenting cells and T cells that may be useful as an additional product for immunotherapy of asthma. Since humans have been naturally exposed to this protein through exposure to *Ascaris* without harmful reactions, this suggests that adverse effects associated with Al-CPI administration would be less probable. Although further deep characterization of its allergenic activity is necessary, preliminary data suggest it has low potential to induce allergic reactions. A graphic integration of the different effects of AL-CPI and the possible mechanisms of action leading to its anti-inflammatory properties is presented in Fig. 6.

Conclusions

The search for better anti-inflammatory compounds is the practical outcome of a long time basic research in the field of immunoparasitology. From epidemiological observations in humans, the field has advanced to the experimentation in animal models and *ex vivo* human cells using single purified molecules and multi-omics tools. In this race, an indefinite number of helminth-derived immunomodulators have been discovered, among them an *A. lumbricoides* cystatin, which has been evaluated by several strategies. Al-CPI is anti-inflammatory and non-toxic in mouse models of intestinal and lung inflammation. In human *ex vivo* experiments, it reduces the mite-induced IL-5 secretion from PBMC and the maturation of monocyte-derived dendritic cells. In addition, Al-CPI is hypoallergenic. These characteristics suggest that it is potentially useful for treating INDs such as intestinal inflammatory disease and asthma, which means that further immunological and pharmacologic studies should be done. In addition, this and other helminth-immunomodulators will help to further explore the basic mechanisms underlying the pathophysiology of IND.

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Conflict of interest. None.

Ethical standards. Not applicable.

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