

Review

Recombinant proteins of helminths with immunoregulatory properties and their possible therapeutic use



Leonardo Nascimento Santos, Luis Gustavo Carvalho Pacheco, Carina Silva Pinheiro, Neuza Maria Alcantara-Neves*

Institute of Health Sciences, Federal University of Bahia, Salvador, BA, Brazil

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ABSTRACT

The inverse relationship between helminth infections and the development of immune-mediated diseases is a cornerstone of the hygiene hypothesis and studies were carried out to elucidate the mechanisms by which helminth-derived molecules can suppress immunological disorders. These studies have fostered the idea that parasitic worms may be used as a promising therapeutic alternative for prevention and treatment of immune-mediated diseases. We discuss the current approaches for identification of helminth proteins with potential immunoregulatory properties, including the strategies based on high-throughput technologies. We also explore the methodological approaches and expression systems used for production of the recombinant forms of more than 20 helminth immunomodulatory proteins, besides their performances when evaluated as immunotherapeutic molecules to treat different immune-mediated conditions, including asthma and inflammatory bowel diseases. Finally, we discuss the perspectives of using these parasite-derived recombinant molecules as tools for future immunotherapy and immunoprophylaxis of human inflammatory diseases.

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1. Introduction

Helminthic parasites, by co-evolution with mammals, developed mechanisms to modulate the immune system of their

hosts in order to survive for long periods and, consequently, cause impact in the development and manifestation of human chronic inflammatory diseases (Ditgen et al., 2014; Bashi et al., 2015; Maizels and McSorley, 2016). The increasing prevalence of immune-mediated inflammatory diseases including asthma, rhinitis, intestinal inflammatory disease, diabetes and several others has been attributed to modern lifestyles in industrialized countries, in particular to the lack of exposure to infectious agents such as microorganisms and parasitic helminths (Strachan, 1989; Parker and Ollerton, 2013; Maizels et al., 2014).

* Corresponding author at: Institute of Health Sciences, Federal University of Bahia, Av. Reitor Miguel Calmon, S/N, Canela, CEP 40110.100 Salvador, Bahia, Brazil.

E-mail addresses: nsleo.1@gmail.com (L. Nascimento Santos), lgcpacheco@gmail.com (L.G. Carvalho Pacheco), carinaspinheiro@yahoo.com.br (C. Silva Pinheiro), neuzalcantara@gmail.com (N.M. Alcantara-Neves).

In fact, helminth infections have been used experimentally to treat inflammatory disorders, with some encouraging results. For example, *Trichinella spiralis* infection has been shown to down-immunoregulate type 1 diabetes, ulcerative colitis and experimental autoimmune encephalomyelitis in experimental models (Ashour et al., 2014; Gruden-Movsesijan et al., 2010; Hübner et al., 2012), whereas *Schistosoma mansoni* was also able to suppress airway inflammation (Pacífico et al., 2009).

Likewise, *Trichuris suis* eggs and alive *Necator americanus* infective larvae have been reported to treat human inflammatory bowel disease and allergic rhinitis in humans (Croese et al., 2006; Summers et al., 2003). However, evaluation of these therapies by most reliable meta-analysis studies concluded that there was not sufficient evidence to ensure their efficacy and safety (Croft et al., 2012; Garg et al., 2014; Maizels, 2016). Thus, prior to considering clinical treatments using these biological products, the helminths or their products require careful selection and the elucidation of mechanisms underlying their immunomodulatory capabilities to avoid the effects related to this ability such as reducing the effectiveness of vaccines, increased susceptibility to co-infection and inhibit the immune response against tumors (Maizels and McSorley, 2016).

Important advances have occurred in understanding the parasite-host relationship, and some molecular and cellular mechanisms are already well defined. These mechanisms include the induction of regulatory cytokine production (IL-10 and TGF- β) (Shiny et al., 2011); CD4+ CD25+ Foxp3+ T cell (Treg cells) recruitment (Layland et al., 2013); alternative macrophage activation inducing an anti-inflammatory phenotype (Du et al., 2014) and an immune response shift (Hübner et al., 2012). However, these mechanisms are variable depending on the parasite species or its products, experimental model, treatment protocol, among other factors (Bashi et al., 2015; Maizels and McSorley, 2016).

Native proteins isolated from helminths have been described as potent immunomodulators. The ES-62 glycoprotein from *Acanthocheilonema viteae* has been shown to interact with several different cells and molecules of the immune system to decrease the inflammatory response, such as in arthritis (Pineda et al., 2012). Ideally, proteins isolated from the parasites could be purified and evaluated for their immunomodulatory potential. However, the processes for obtaining the native molecules are often laborious, time consuming and not effective for production complying with required purity standards, reproducibility, and in large-scale to biophysical and biochemical characterization and clinical use (Fernández-Robledo and Vasta, 2010; Vedadi et al., 2010).

These proteins can be produced through recombinant DNA technology in different expression systems such as bacteria, yeast, protozoa, insect cells and mammalian cells (Frenzel et al., 2013). Some parameters such as growth characteristics, folding, purification and posttranslational modifications are crucial for the choice of the expression system (Pfeifer and Khosla, 2001) and are likely to have an impact on biological activity, stability and pharmacokinetics (Fujihara et al., 2008).

Despite the high cost, most of the recombinant protein pharmaceuticals currently in use are produced in mammalian cell lines, because folding, secretion and posttranslational modifications are more similar to those of human native proteins, a requirement particularly important to proteins whose suitable glycosylation is essential for biological activity (Frenzel et al., 2013). For example, the most therapeutic glycoproteins require suitable sialylation, which is more likely to be achieved by mammalian cells, despite this expression systems have disadvantages such as heterogeneity of glycans and, particularly, handling difficulties to produce specific changes (Hamilton et al., 2006; Kallolimath et al., 2016). In addition, other eukaryotic expression systems as yeast have advantages such as higher yield, shorter fermentation times, growth in

chemically defined media and, especially, ease of handling which provides flexibility to insert appropriate biochemical machinery for required post-translational modifications (Hamilton et al., 2006; Schaefer and Plückthun, 2012). These characteristics can be useful for the biosynthesis of helminth recombinant proteins, which are not yet well characterized biochemically and demand specific post-translational modifications.

On the other hand, *E. coli* expression systems have been most useful in producing helminth recombinant proteins to test for immunomodulation with some encouraging results in vitro and in experimental models. This preference is due to the advantages of low cost, ease of handling and high yielding, despite the lack of post-translational modifications and inappropriate folding that may be important for their biological function (Anné et al., 2012; Assenberg et al., 2013).

2. Rational strategies for obtaining recombinant helminth molecules for therapeutics or immunoprophylaxis purposes

Nowadays, multi-technological approaches involving parasitology, genomics, transcriptomics and proteomics methods, associated with a variety of bioinformatics strategies, have contributed enormously to the identification of helminth proteins with potential immunomodulatory properties (Du et al., 2011; Cantacessi et al., 2011; He et al., 2014; Ebner et al., 2014).

Several in vivo techniques using established experimental animal models for asthma (Baqueiro et al., 2010; Navarro et al., 2016), colitis (Brenna et al., 2013) and autoimmune diabetes (Viehmam Milam et al., 2014) are currently available to test helminthic molecules for further use as immunotherapeutic agents of human inflammatory diseases when their mechanisms of action and safety became well recognized.

Many genomic sequences of helminth parasites that are considered to modulate the mammalian host's immune system have become recently publicly available; these include the genomes of *Ascaris suum* (Jex et al., 2011), *Brugia malayi* (Ghedini et al., 2007), *Fasciola hepatica* (Cwiklinski et al., 2015), *Necator americanus* (Tang et al., 2014), *Schistosoma mansoni* (Berriman et al., 2009), *Strongyloides* spp (Hunt et al., 2016), *Toxocara canis* (Zhu et al., 2015), *Trichinella spiralis* (Mitrevva et al., 2011), *Trichuris suis* (Jex et al., 2014), and *Trichuris trichiura* (Foth et al., 2014). A number of proteins have already been predicted through *in silico* analysis of these genomes that could be considered as potential immunomodulatory molecules for further evaluation (Ghedini, 2014; Foth et al., 2014; Jex et al., 2014).

Through transcriptomics, using high-throughput sequencing technologies, it is now possible to study all the protein-encoding transcripts produced by specific helminthic tissues during different developmental stages of these parasites (Choi et al., 2011) and also during contact with the host (Foth et al., 2014), allowing for the evaluation of genes involved in different cellular functions and mechanisms such as immunomodulation (Gasser, 2013). The integration of the information gathered from transcriptomic studies with data obtained at the proteomic level will not only contribute toward improving our understanding of parasite biology and of the host-parasite interplay, but might also aid in the identification of novel immunotherapeutic helminthic molecules. For this, novel dedicated databases and bioinformatic pipelines for integrated analysis of helminth “-omics” data, such as the WormBase Parasite, HelminDB and Heminth.net, were made available in recent years (Mangiola et al., 2013; Martin et al., 2015; Howe et al., 2016; Korhonen et al., 2016).

Fig. 1 shows a diagram describing the steps for a general approach to identify helminthic molecules for immunotherapy and immunoprophylaxis of inflammatory diseases. The informa-

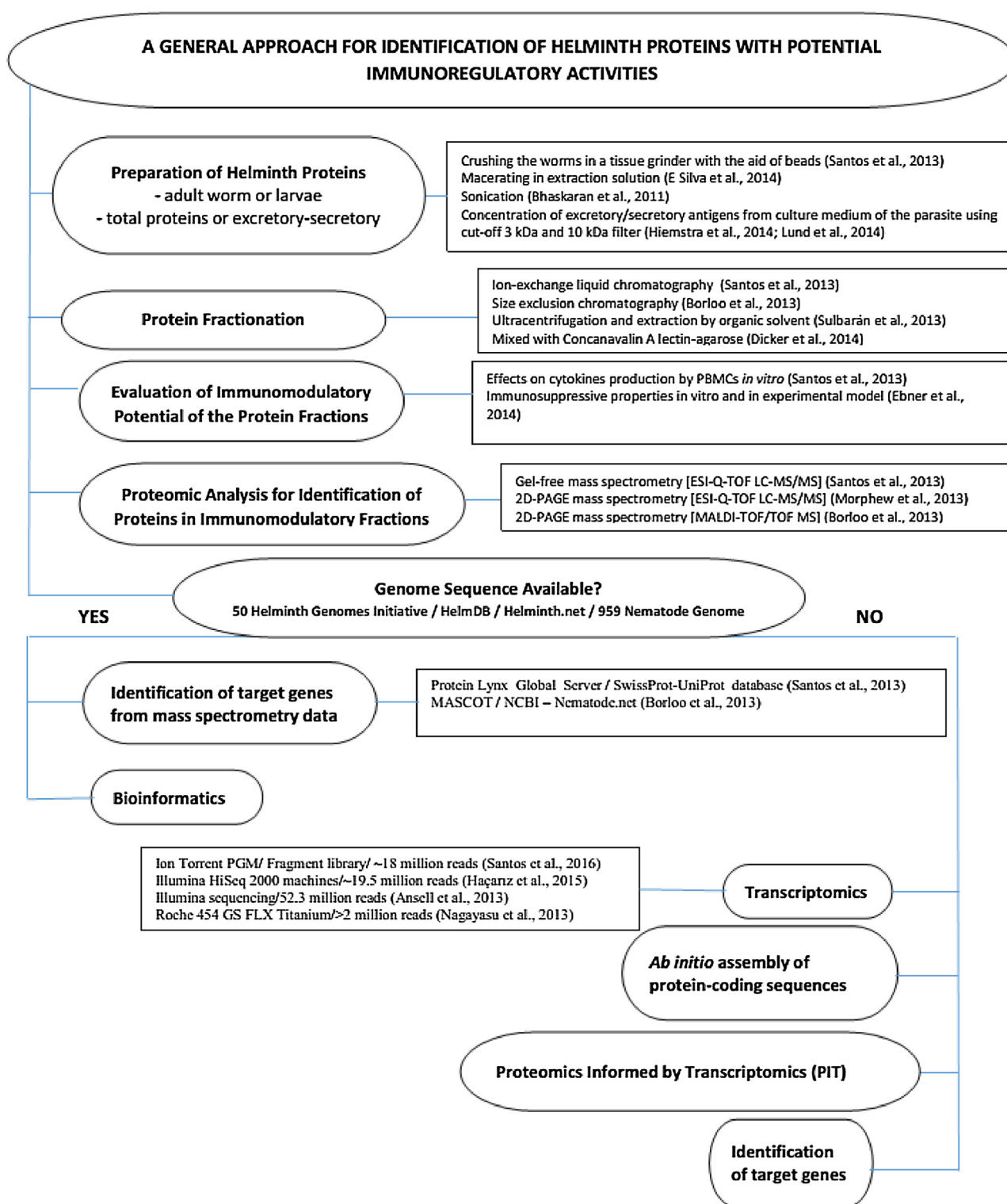


Fig. 1. A general approach for identification of helminth proteins with potential immunoregulatory activities.

tion was extracted from genomic, transcriptomic and proteomic methods to identify target genes and *in vitro* tests of the produced target molecules, making use of different bioinformatics tools for deeper exploration of the currently available helminthic-related public databases. In fact, there is a growing number of recombinant proteins derived from helminth parasites described in the literature that presents some immunoregulatory activity that are being

tested for the inhibition of several immunological disorders (Santos et al., 2013).

In this way, the aim of this review is to discuss the current approaches for identification of helminth proteins with potential immunoregulatory properties, to explore the methodological approaches and expression systems that have been used for their production and discuss their performances when evaluated as immunotherapeutic molecules to treat different immune-

mediated conditions, including asthma, other allergies, rheumatoid arthritis and inflammatory bowel diseases.

3. Different classes of recombinant helminth proteins with therapeutic potential for immune-mediated diseases

3.1. Protease inhibitors

Many proteases are produced during inflammation and play important roles in inflammatory processes. Recently, the involvement of protease-activated receptors (PAR) in inflammatory diseases has been studied, and it was demonstrated their presence in several types of immune and structural cells (Zhang et al., 2014). Evidence of their participation has been raised in human allergic rhinitis (Lee et al., 2007). In TNBS-induced colitis, a synthetic PAR-2 antagonist prevented mucosal damage, inflammation and edema, even more than the clinically used drug, sulfasalazine (Lohman et al., 2012). Antagonists of PAR or inhibitors of the proteases that activate this receptor may be valuable therapy candidates for inflammatory diseases.

In fact, parasite protease inhibitors such as the cystatins (cysteine protease inhibitors) have shown promising results as immunoregulatory agents. In this sense, a filarial recombinant cystatin (AvCystatin) exhibited a modulatory response in mice with pollen-induced allergy, through IL-10 production and consequently decreased Th2-response, essential to IgE class switching, antibodies which play a central role in the establishment of allergic reactions. In addition, other important players of allergy inflammation such as mast cell protease and mast cells recruitment were reduced (Daniłowicz-Luebert et al., 2013).

More recently, a novel type of regulatory macrophage generated by stimulus with that recombinant protein (AvCystatin) was identified by phenotyping. The adoptive transference of these cells was able to suppress allergic airway inflammation, and when in co-culture with dendritic and CD4⁺ T cells, these macrophages induced IL-10 production (Ziegler et al., 2015). This protein was expressed in *Escherichia coli* as a biologically active cystatin, after it was identified by screening a cDNA expression library from *Acanthocheilonema viteae* female mRNA, using sera of rodents resistant to challenge infection, after vaccination with the larvae of this nematode (Hartmann et al., 1997).

A 17 kDa cystatin from another filarial parasite, *Onchocerca volvulus*, was produced by *E. coli* after cloning by PCR from a cDNA library of infective larvae of *O. volvulus* and purified under non-denaturing conditions by affinity chromatography. This functionally active recombinant cystatin (named rOv17 or onchocystatin) was able to inhibit the human PBMC proliferation and increase the IL-10 production, which was related with diminishing expression of surface costimulatory molecules on T cells (Schönemeyer et al., 2001).

Another recombinant protein produced by *E. coli*, a cystatin derived from *Angiostrongylus cantonensis* (AcCystatin), was able to inhibit several inflammatory parameters such as expression of chemokines and cytokines, serum IgE levels and lung inflammation in an ovalbumin/aluminium hydroxide-induced rat model of asthma, when used for immunization or treatment (Ji et al., 2015). Its ortholog from *Schistosoma japonicum*, a type II cystatin (SjCystatin) was identified through the analysis of an online database and transformed into *E. coli*, mostly expressed in a soluble form. This protein was functional and able to regulate macrophage inflammatory response by inhibiting TNF- α , IL-6 and nitric oxide. In addition, it induced IL-10 production by alternatively activated macrophages leading them to present an immunomodulatory phenotype (Yang et al., 2014). In addition, another cystatin (family I) from *S. japonicum* was expressed in *E. coli* and was effective in diminishing the

severity of 2, 4, 6-trinitrobenzene sulphonic acid (TNBS)-induced colitis through a reduction of the Th1 response associated with enhanced Treg and Th2 responses (Wang et al., 2016).

Another strategy was adopted to identify a coding gene for a cysteine protease inhibitor from *Heligmosomoides polygyrus*. The corresponding gene was amplified from a cDNA library as template, using primer sequences based on conserved sequences of cystatins previously described for helminths in the GenBank (Sun et al., 2013). The recombinant protein produced in *E. coli* was functional and able to suppress dendritic cells activation through reduction in MHC-II expression. The TLR-induced expression of the co-stimulatory molecules CD40 and CD86 was reduced when these cells were treated by this helminth protein (Sun et al., 2013).

Also taking advantage of the similarity with known helminthic cystatin, a gene sequence encoding cystatin of the fluke worm *Clonorchis sinensis* (CsStefin-1) was identified by analysis of a cDNA library of the adult worms and then expressed in *E. coli*, resulting in expression of functional protein related to family 1 stefins. Phylogenetic analysis revealed that the protein had structural similarity to human stefin B (Kang et al., 2011). This recombinant Cs-Stefin-1 reduced dextran sodium sulphate (DSS)-induced murine colitis, through increasing IL-10 production by macrophages which seems to be caused by ERK phosphorylation (Jang et al., 2011).

3.2. Proteases

Although causing an opposite effect to that of protease inhibitors, proteases may also display immunoregulatory activities. The most common cysteine proteases found in parasites are those from the family of cathepsins B and L; however, many of these molecules are secreted, in contrast to mammalian cathepsins, which are stored in lysosomes. These proteases have a variety of functions that are essential to parasitism, including immunomodulation and parasite evasion of the immune system (Smooker et al., 2010).

The secreted cathepsins are expressed as proenzymes, which are converted into their active forms after cleavage, self-catalyzed or not. However, this step did not impair the synthesis of the active form by recombinant technology. The cathepsins L1 of *Fasciola hepatica* and B1 of *S. mansoni* were produced in their active forms, starting from the encoding cDNA for wild-type preprotein, in *Pichia pastoris* (Collins et al., 2004; Donnelly et al., 2010; Stack et al., 2005). In addition, cathepsin L1 from *F. hepatica* produced by *P. pastoris* was much more stable at pH 7 and 37 °C than human cathepsin L (Collins et al., 2004); it is noteworthy to mention that stability is an important feature of a product for clinical use.

In a murine sepsis model, the administration of cathepsin L1 of *F. hepatica* (FhCL1) protected mice from death through suppression of inflammation by decreasing IL-12, TNF- α , and IFN- γ levels. This suppression seems to have been caused by direct degradation of TLR3 and inhibition of macrophage activation through the TRIF-dependent and Myd-88-independent signaling pathway. Likewise, cathepsin B1 from *S. mansoni* had a similar effect (Donnelly et al., 2010).

3.3. Cytokine homologs

Macrophage migration inhibitory factor (MIF) is a human cytokine involved in innate immunity and is a stimulator of pro-inflammatory mediator production (Roger et al., 2003). Meanwhile, the type-1 and type-2 MIF homologs are molecules produced by several helminths as part of their machinery to evade the host immune system, which reflects in their hosts immune modulation (Vermeire et al., 2008). For example, the recombinant type-2 MIF homolog from *Anisakis simplex* third-stage larvae (rAs-MIF) expressed in *E. coli*, was able to inhibit Th1 and Th2 cytokine pro-

duction in intestinal and airway inflammation, respectively. In both disorders, the immunoregulation was correlated to CD4⁺ CD25⁺ Foxp3⁺ T cells recruitment together with increased IL-10 and TGF- β production in bronchoalveolar lavage fluid or in the spleens and mesenteric lymph nodes (Cho et al., 2011; Park et al., 2009).

In DSS-induced intestinal inflammation, rAs-MIF promoted improvement in signs associated with intestinal inflammation such as reduction of DAI (disease activity index), amelioration of histopathology and absence of colon shortening, which is a marker of severe large intestine inflammation (Cho et al., 2011). Treatment of murine epithelial cell lineage (CT-26) cultures with rAs-MIF was associated with phosphorylation of both ERK and p38 pathways, with inhibition of IL-10 expression when using ERK-inhibitor (Cho et al., 2011).

The *A. simplex* MIF-like coding gene (As-MIF) was isolated randomly from a cDNA library of *A. simplex* third-stage larvae in a study of expressed sequence tags (ESTs) and was then successfully expressed in *E. coli*; in the purified protein, the active site of tautomerase enzyme activity appeared to be located around Pro2 residue as a catalytic residue (Park et al., 2009).

A MIF-like coding gene of *S. ratti* was also identified by ESTs analysis, and expressed in a bacterial system. Phylogenetic analysis using the protein sequence showed that Sra-MIF is related to type-2 MIF. Recombinant Sra-MIF was able to induce IL-10 production by human peripheral blood mononuclear cells in vitro (Younis et al., 2011a).

3.4. Heat shock proteins

Heat shock proteins (HSPs) are evolutionary conserved proteins expressed by prokaryotic and eukaryotic organisms, including mammals. Sra-HSP-17, for example, is an immunoregulatory protein of *S. ratti* that more expressed in the adult female parasites than in the infective third-stage larvae. Peptides characterized by ESTs related to HSPs occur abundantly in the excretory/secretory products of female parasites, including HSP17s (Younis et al., 2011b). The recombinant Sra-HSP17 produced in *E. coli* induced IL-10 production by human PMBCs without causing any change in TNF- α production. Interestingly, IgG antibodies from rats immunized with the recombinant protein recognizing native Sra-HSP-17, as well as its recombinant protein (Younis et al., 2011b), demonstrated the conservation of epitopes in the recombinant form, which may be essential for its activity in the immune system. Further, the small *Strongyloides* HSP10 appears to contribute in parasite-host interaction (Tazir et al., 2009).

Likewise, recombinant HSP60 of *Wolbachia*, an endosymbiont bacterium, present in many filarial nematodes, was produced in *E. coli* following amplification of its coding sequence from *B. malayi* genomic DNA. Both rHSP60, as well as the *B. malayi* adult worm extract were able to suppress lymphocyte proliferation PBMCs of filarial infected-patients, suggesting that this protein may contribute to the host immunoregulation promoted by filarial parasites (Shiny et al., 2011).

3.5. Lectins

Lectins are proteins that bind to specific carbohydrate structures and can thus recognize particular glycoconjugates. Galectins are S-type lectins with affinity for β -galactoside. Galectin encoding cDNA was identified by immunoscreening a *Haemonchus contortus* cDNA library with antisera of lambs vaccinated with a galactose-containing glycoprotein complex, and was then used for recombinant protein production in *E. coli* (Newlands et al., 1999).

Vaccination with affinity-purified recombinant galectin did not confer protection against infection to lambs, but it induced partial protection to goats (Newlands et al., 1999; Yanming et al., 2007).

In addition, it was found that the recombinant galectins of male and female *H. contortus* (rHco-gal-m and rHco-gal-f, respectively), produced in the bacterial expression system, were able to cause several events related to down-modulation of the host immune system shown in goat PBMC cultures. The Concanavalin A-induced production of cytokines IL-1 β , IL-4, IL-6 and IFN- γ was reduced when the cells were treated by rHco-gal-m and rHco-gal-f; likewise, expression of molecules important for the T cell development pathway and proteins involved in lymphocytes migration were down-regulated (Wang et al., 2014).

A recombinant galectin-9 homolog (rTI-gal) of the canine roundworm, *Toxascaris leonina*, obtained from the female adult worm cDNA was also produced in *E. coli*. Its amino acid sequence shared more than 80% identity with filarial nematode galectins and 35% identity with human galectins (Kim et al., 2010). When tested in a mouse model of dextran-induced colitis, it promoted the inhibition of intestinal inflammation via enhanced IL-10 and TGF- β production associated with decreased IL-4 and IFN- γ levels. Regarding intracellular signaling pathways, rTI-gal induced the ERK 1/2 and p38 phosphorylation in intestinal epithelial cells, wherein the elevated expression of TGF- β was directly related to the p38 pathway activation (Kim et al., 2010).

3.6. Other proteins

OvALT and OvNLT, recombinant proteins derived from the excretory/secretory products of *Onchocerca volvulus* whose recombinant forms were also obtained in a *E. coli* system were able to suppress antigen-specific T-cell proliferation and to decrease IL-2 and IFN- γ secretion related to apoptosis, even after heat inactivation and digestion by proteases. This finding indicates that these suppression was promoted by these protein fragments once the effect of LPS or low-weight molecular contaminants was excluded (Hartmann et al., 2013).

Another excretory/secretory product, a polyprotein (DiAg) from the canine filarial parasite *Dirofilaria immitis*, also obtained in the recombinant form using *E. coli* as host; the recombinant product (rDiAg) inhibited the immune-mediated pregnancy loss in a murine model mimicking human pregnancy failure, through decreased IL-4, IL-23 and TNF- α concentrations in serum from treated mice (Komine-Aizawa et al., 2011; Tezuka et al., 2003).

rBmAsnRS is the recombinant form of an asparaginyl-tRNA synthetase excretory/secretory protein of *Brugia malayi* that induced the upregulation of the IL-10 receptor and had an effect on human gene expression distinct from IL-8, despite being structurally related to this cytokine, suggesting a potential anti-inflammatory effect; production of this molecule in *E. coli* resulted in soluble and enzymatically active protein (Kron et al., 2013). In experimental colitis, rBmAsnRS succeeded in decreasing cellular infiltrate in the lamina propria as well as restoring normal bowel wall thickness and crypt formation. At the molecular level, increased IL-10 and IL-4 production was associated with decreased IL-17 and IFN- γ levels (Kron et al., 2013). Therefore, this effect enables its use as a potential immunomodulatory therapy tool.

Ts-specific 53 kDa glycoprotein (TsP53) is an excretory/secretory antigen of *Trichinella spiralis* larvae (Du et al., 2011). The recombinant protein produced in *E. coli* was able to ameliorate TNBS-induced colitis, resulting in a reduction in the most evident injuries in colitis such as mucosal and epithelium damage in the treated group. With regard to the humoral immune response, the TsP53-treated group produced specific IgG1 (related to Th2 response) but not IgG2a (related to a Th1 response). Consistent with the Th2 profile induced by treatment, the serum levels of IL-4 and IL-13 were elevated, while the levels of TNF- α and IFN- γ were diminished (Du et al., 2011). Two secreted proteins from *Strongyloides ratti* were reported to affect the mucosal homeostasis

by cytokine release and promoting epithelial wound healing which would be of relevance in anti-inflammatory activities of helminth molecules (Anandarajah et al., 2016; Ditgen et al., 2016).

Sm16/SmSLP is a 16 kDa anti-inflammatory protein from *S. mansoni*, whose recombinant production from the native cDNA has very low yields when using bacteria, yeast and insect systems (Valle et al., 1999). An alternative was to construct a codon-optimized synthetic amino acid encompassing residues 23–117 of the corresponding Sm16-encoding gene to produce modified and soluble protein to facilitate the expression and purification, especially due to replacing Ile-92 and Leu-93 with Ala residues. Thus, the modified protein was successfully produced by *E. coli* and *P. pastoris* with a deduced molecular mass of 13.6 kDa, which seems to form a 9-subunit oligomer. It inhibited LPS-induced production of IL-6, TNF- α and IL-1 β in whole blood, as well as avoided TLR4 and TLR3 activation and delayed antigen processing on murine bone marrow-derived macrophages (Brännström et al., 2009; Sanin and Mountford, 2015).

Sm16 shares 99% of similarity with its homolog from *S. japonicum* (Sj16). A recombinant Sj16 was produced in *E. coli* (Hu et al., 2009). Sun et al. (2010) showed that recombinant Sj16 protein presented a similar effect as dexamethasone (a steroid hormone) in CFA-induced arthritic rats; it inhibited inflammatory cell infiltration and pannus formation of ankle joint tissue in histological examinations. In addition, rSj16 was able to inhibit TNF- α , IL-1 β and decreased nitric oxide presence in serum, whereas serum IL-10 levels recovered as before arthritis induction (Sun et al., 2012).

Recombinant proteins of the parasitic flatworm *S. mansoni* (Sm22.6 and Sm29) also produced by *E. coli* were able to suppress ovalbumin-induced airway inflammation in a murine model during a standard immunization/sensitization protocol. Interestingly, the immunomodulation occurred by recruitment of CD4⁺ CD25⁺ Foxp3⁺ T cells together with increased IL-10 production for Sm22.6 but not for Sm29. According to the results observed for injection of *S. mansoni* eggs in an airway allergic inflammation model, the increase of IL-10 expression was not essential for the suppression of inflammation (Pacífico et al., 2009).

An overview of the herein cited and discussed helminth recombinant proteins with immunoregulatory effects is presented in Table 1.

4. Perspectives for the use of recombinant helminth molecules for immunotherapy

Currently, recombinant helminth proteins with immunomodulatory potential have been produced in yeast *P. pastoris* and mostly in bacterial (*E. coli*) expression systems with some promising results. However, there are still a myriad of improvements necessary in expression systems to generate a product that more closely resembles the native protein. For example, alternative expression hosts could be explored to express heterologous proteins that have suitable structures (Fernández and Vega, 2013), to enhance the immunoregulation achieved by these molecules.

The development of novel expression systems more phylogenetically close to nematodes, which presumably provide the correct folding and post-translational modifications, could prove valuable to aid the development of molecules suitable for novel human immunotherapies. The free-living nematode *Caenorhabditis elegans* has already been used as a protein expression host and a glycosylated cathepsin L from *Haemonchus contortus* was successfully expressed in this system, in an active form (Murray et al., 2007).

The viability of *C. elegans* as an expression system comes from an in-depth knowledge of its genetics, considering that this is the best-characterized nematode. Furthermore, an important feature for heterologous protein synthesis is its ability to perform the addi-

tion of phosphorylcholine as a posttranslational modification that is a common attribute of helminths and is involved in some of their immunomodulatory actions (Deehan et al., 2002; Grabitzki et al., 2008).

An alternative to developing novel expression systems is the optimization of the currently available systems, for example, by engineering the host for more adequate protein production. In fact, the functional transfer of the *Campylobacter jejuni* glycosylation machinery in *E. coli* enabled it to produce glycosylated proteins (Schwarz et al., 2010). Pathogenic bacteria possess the machinery capable of promoting the addition of phosphorylcholine as a post-translational modification (Müller et al., 2014) that, in the future, could be exploited for obtaining proteins with this immunomodulatory moiety.

Another strategy is to rely on parasitic molecules for the prospection of synthetic drugs able to reproduce the effects or enhance them. The development of ES-62-analogues based on the phosphorylcholine moiety have been successfully used in treatment of collagen-induced arthritis in mice and nephritis in systemic lupus erythematosus model, even though native ES-62 is not suitable as drug due to its immunogenicity (Pineda et al., 2015; Rodgers et al., 2015).

The immunogenicity is an actual concern about use of therapeutic recombinant proteins, particularly because heterologous systems provide different posttranslational modifications that provoke changes in protein structure and favor the immune system identify the molecules as non-self (Jefferis, 2016). Despite protein glycosylation is a posttranslational modification more prevalent and conserved in yeast, insect cells, plants and mammals, there are a lot of differences in glycosylation patterns, even between mammalian cell lines (Croset et al., 2012). A promising alternative to overcome this problem has been to genetic engineering of glycosylation pathways in yeast to obtain homogeneous and humanized N-glycans (Hamilton et al., 2006; Arico et al., 2013).

Current development of DNA sequencing platforms associated to new bioinformatics tools have achieved great progress in helminth genomics, particularly, in the search for molecules with the potential to down-regulate the host's immune system. For example, possible proteins responsible for the immunomodulatory effects of fractions from *Trichuris trichiura* extract were identified by our group using a proteomics approach (Santos et al., 2013); then, a transcriptomic analysis contributed to effective identification of these proteins and, additionally, permitted the identification of protein orthologs of other proteins which have already been shown to possess immunomodulatory properties (Santos et al., 2016).

Previously, the first transcriptome of *Trichuris suis* also provided a number of molecules likely to be involved in immunomodulation, considering the effect of this parasite on inflammatory bowel disease (Cantacessi et al., 2011). In addition, the draft genome assembly for *T. trichiura* and the reference genome and transcriptome for the mouse parasite *T. muris* provided considerable subsidies for the *Trichuris* genomic databases and identified genes differentially expressed by sex and morphological region, which have great importance for the understanding of host-parasite relationship (Foth et al., 2014).

In addition, a proteomics analysis of the excretory/secretory component of *Ancylostoma caninum* identified a number of proteins belonging to several classes which are expected interacting with the host such as metalloproteases, cysteine proteases, lysozyme-like and lectins (Mulvenna et al., 2009). In particular, helminth secreted lectins have known participation at the host immune system evasion (Loukas and Maizels, 2000).

Therefore, studies with the purpose of selecting proteins with immunomodulatory activities and comparing the native proteins to respective recombinant forms and their variants would be an excellent approach to the optimization of recombinant proteins in order

Table 1
Recombinant helminth proteins with immunoregulatory properties and strategies for their production.

Recombinant protein	Helminth Source	Protein Class	Immunoregulation mechanism	Experimental model	Expression Host System/Vector/Induction	Molecular weight	Reference
protease cathepsin L (rFheCL1)	<i>F. hepatica</i>	Protease	Suppression macrophage-driven Th1 inflammation	Murine sepsis model; Macrophage Cell Culture	<i>P. pastoris</i> GSII5/pPIC9K/ Methanol	24.5 kDa	Collins et al. (2004); Donnelly et al. (2010)
Cathepsin B1 (SmB1)	<i>S. mansoni</i>	Protease	Suppressing macrophage proinflammatory cytokine secretion	Macrophage Cell Culture	<i>P. pastoris</i> GSII5/pPIC9K/ Methanol	39.0 kDa	Donnelly et al. (2010); Stack et al. (2005)
sigma class glutathione transferase (rFhGST-si)	<i>F. hepatica</i>	Antioxidant	Suppressing Th17 immune response	Dendritic Cells Culture	<i>E. coli</i>	24.5 kDa	Dowling et al. (2010)
Type I cystatin (CsStefin-1)	<i>C. sinensis</i>	Cystatin	Recruitment of macrophages IL-10 secreting	DSS-induced Intestinal inflammation	<i>E. coli</i> (M15)/pQE-30/ IPTG	12.0 kDa	Jang et al. (2011)
(rHp-CPI)	<i>H. polygyrus</i>	Cystatin	Modulate DC maturation and activation	Dendritic Cells Culture and Co-culture with T cells	<i>E. coli</i> (Origami)/pET32a/ IPTG	15,7 kDa	Sun et al. (2013)
AvCystatin	<i>A. viteae</i>	Cystatin	Suppression of allergen-specific Th2 responses	Grass pollen-specific allergic response	<i>E. coli</i> pET28a/ IPTG	17.2 kDa	Daniłowicz-Luebert et al. (2013); Hartmann et al. (1997)
FhHDM-1	<i>F. hepatica</i>	Cathelicidin-like	It binds to and neutralizes LPS	LPS-induced inflammation	<i>E. coli</i> (BL21)/pET21b/ IPTG	9.2 kDa	Robinson et al. (2011)
53 kDa protein (rTsP53)	<i>T. spiralis</i>	glycoprotein	Th2 immune deviation	TNBS-induced colitis	<i>E. coli</i> (BL21)/pET28a/ IPTG	53.0 kDa	Du et al. (2011)
Bm-33	<i>B. malayi</i>	Pepsin inhibitor	Decrease lymphoproliferation	PBMC of individuals infected by <i>W. bancrofti</i>	<i>E. coli</i> BL21 (DE3)/ pRSET-A/IPTG	33.0 kDa	Krushna et al. (2011)
rDiAg	<i>D. immitis</i>	Polyprotein	IL-4, IL-23, TNF- α and IL-17 inhibition	Immune-mediated pregnancy loss	<i>E. coli</i> HMS174 (DE3)/ pET3A/IPTG	15.0 kDa	Komine-Aizawa et al. (2011)
Sra-MIF	<i>S. ratti</i>	Cytokine homolog	Increasing IL-10 level	PBMC culture	<i>E. coli</i> BL21/pJC45/ IPTG	13.5 kDa	Younis et al. (2011a)
Sj16	<i>S. japonicum</i>	–	Increasing IL-10 and Inhibition proinflammatory cytokines production and NO	Macrophage cell Culture	<i>E. coli</i> BL 21 (DE3)/ pGEX-4T/IPTG	16.0 kDa	Sun et al. (2012)
ShTSP2	<i>S. haematobium</i>	Protein of the tegument	Increasing IL-10 level	–	<i>E. coli</i>	–	Lima et al. (2013)
Sm29	<i>S. mansoni</i>	Membrane-bound glycoprotein	Increasing IL-10 level	–	<i>E. coli</i> BL21(DE3)/ pET21a/IPTG	18.0 kDa	Lima et al. (2013)
As-MIF	<i>A. simplex</i>	Cytokine homolog	Increasing IL-10 and TGF- β level and Treg Recruitment	Intestinal colitis and Airway allergic inflammation	<i>E. coli</i> BL 21 (DE3)/ pET28a/IPTG	13.0 kDa	Cho et al. (2011); Park et al. (2009)
rHco-gal-m rHco-gal-f	<i>H. contortus</i>	Lectin	Modulation of several signaling cascades <i>in vitro</i>	PBMC of healthy goats culture	<i>E. coli</i> DH5 α /pBV220/ IPTG	32.5 kDa	Wang et al. (2014)
Annexin B (rCsANXB30)	<i>C. sinensis</i>	Binding protein	High induction of IL-10 and IgG1	–	<i>E. coli</i> BL21(DE3)/pET28a/ IPTG	38.8 kDa	He et al. (2014)
Sra-HSP17s	<i>S. stercoralis</i>	Heat shock protein	Induction IL-10 production, but not of TNF- α	PBMC of healthy individuals Culture	<i>E. coli</i> BL21(DE3)/ pJC45/IPTG	18–19 kDa	Younis et al. (2011b)
OvALT-2	<i>O. volvulus</i>		Induction of T cell death	Spleen cell culture	<i>E. coli</i> BL21(DE3)/ pJC40/IPTG	15.0 kDa	Hartmann et al. (2013)
OvNLT-1	<i>O. volvulus</i>		Induction of T cell death	Spleen cell culture	<i>E. coli</i> BL21(DE3)/ pJC40/IPTG	–	Hartmann et al. (2013)
Galectin-9	<i>T. leonina</i>	Lectin	Increasing production of TGF- β and IL-10.	DSS-induced intestinal inflammation	<i>E. coli</i> BL21/pET28a/ IPTG	31.5 kDa	Kim et al. (2010)
HSP-60	<i>Wolbachia</i>	Heat shock protein	Increasing production of TGF- β and IL-10 and reduced expression of IFN- γ	PBMC of Filariasis Patients Culture	<i>E. coli</i> BL21(DE3)/ pRSET-A/IPTG	60.0 kDa	Shiny et al. (2011)

to reach or even surpass the activity promoted by native molecules and complex extracts.

5. Conclusions

Molecular biology tools have contributed to the identification of helminth proteins responsible for down-modulating their host's immune system. The strategies for their identification and production are diverse, from random discovery to the active and rational search for immunoregulatory molecules, together with the development of suitable expression systems, mostly by different strains of *E. coli*. Many recombinant proteins were produced in their active forms and have mirrored the biological effects observed in infection with live worms.

Although notorious the relevance of some posttranslational modification for immunomodulatory activity of molecules from parasites such as insertion of the phosphorylcholine moiety, in many cases they do not seem essential. Around helminth recombinant protein described, two proteases were produced by *P. pastoris* probably to take advantage of efficient secretion of correct folded form, since glycosylation does not seem important to activity of these proteins, while all other recombinant proteins cited here were produced by *E. coli* and were efficient in perform immunomodulatory activities. Thus, setting a great choice of expression system, given that does not insert posttranslational modifications that might be unnecessary and potentially immunogenic.

In experimental models of inflammatory disease, representing different immune responses, the helminth recombinant proteins have shown promising effects by using the aforementioned mechanisms of immune down-regulation, such as such as inducing anti-inflammatory and reducing pro-inflammatory cytokine production, regulatory cell recruitment and immune deviation, supporting the advance in studies to complete their characterization.

The therapeutic potential of recombinant helminth proteins can be further elevated by overcoming the limitations of current expression systems to obtain molecules more similar to the native protein that would be able to replicate the immunomodulation observed in helminth infections. Besides, prior to considering clinical treatments using these recombinant proteins, experimental treatment protocols should be further investigated to establish the safety of the using as well as the timing and duration of the therapeutic effect.

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