Review article

Failure of in vitro-cultured schistosomes to produce eggs: how does the parasite meet its needs for host-derived cytokines such as TGF-β?

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Abstract

When adult schistosome worm pairs are transferred from experimental hosts to *in vitro* culture they cease producing viable eggs within a few days. Female worms in unisexual infections fail to mature, and when mature adult females are separated from male partners they regress sexually. Worms cultured from the larval stage are also permanently reproductively defective. The cytokine transforming growth factor beta (TGF-β) derived from the mammalian host is considered important in stimulating schistosome female worm maturation and maintenance of fecundity. The means by which schistosomes acquire TGF-β have not been elucidated, but direct uptake in vivo seems unlikely as the concentration of free, biologically active cytokine in host blood is very low. Here we review the complexities of schistosome development and male-female interactions, and we speculate about two possibilities on how worms obtain the TGF-β they are assumed to need: (i) worms may have mechanisms to free active cyokine from the latency-inducing complex of proteins in which it is associated, and/or (ii) they may obtain the cytokine from alpha 2-macroglobulin (A2M), a blood-borne protease inhibitor to which TGF-β can bind. These ideas are experimentally testable.

Keywords: Schistosome; *Schistosoma mansoni*; Egg production; reproduction; TGF-β; Alpha 2-macroglobulin; Alzheimer's disease

1. Introduction

1.1. Schistosomes and schistosomiasis

Schistosomes are digenetic trematode parasites requiring two different hosts: a vertebrate definitive host for sexual reproduction and an aquatic or amphibious snail intermediate host in which to reproduce asexually. Disease in humans is attributable to three main species: *Schistosoma mansoni*, *Schistosoma haematobium* and *Schistosoma japonicum*. The schistosome life cycle in the definitive host begins with free-swimming cercariae that have been released by infected snails locating the host, most likely by chemo-attraction (Freitas et al., 2007; Haeberlein and Haas, 2008), and penetrating through intact skin using potent proteolytic enzymes and muscular activity (McKerrow and Salter, 2002; Curwen and Wilson, 2003; Dvorak et al., 2008). A quantitative histological investigation of *S. mansoni* infections of mice indicated that following skin penetration the young parasites (schistosomula) penetrated blood capillaries (sometimes lymph vessels) and thereafter followed an entirely intravascular route of travel, being carried in the direction of blood flow (Wheater and Wilson, 1979). From about 7 days after skin penetration, *S. mansoni* schistosomula become detectable in the liver and numbers in this organ increase for the next 10-14 days (Doenhoff et al., 1978; Wheater and Wilson, 1979). During this period the young worms increase in size.

Schistosomes (together with some Didymozoidae – trematode parasites of fish) are dioecious and exceptional within the Digenea as all other members are hermaphroditic. From about 25 days after infection, *S. mansoni* male and female worms become morphologically distinguishable and between days 25 and 28 they begin to pair up with the muscular male clasping the more slender female. Further maturation occurs and egg production in infected mice begins between 30 and 35 days p.i. (Doenhoff et al., 1979). Worm development is asynchronous, but ~45 days after the initial infection pairing between male and available

female worms is complete, although there are generally more males than females (Beltran and Boissier, 2010). Maturation of *S. mansoni* worms is impaired in the absence of host-derived factors such as IL-7 (Wolowczuk et al., 1999), thyroid hormones (Saule et al., 2002) and elements of the adaptive immune system (Harrison and Doenhoff, 1983; Hernandez et al., 2004; Riner et al., 2013).

Adolescent male and female worms meet in the hepatic portal vein and after pairing they migrate against blood flow towards capillary beds in the intestine (*S. mansoni* and *S. japonicum*) or further to those of the urino-genital tissues (*S. haematobium*). Each mature female *S. mansoni* worm produces ~300-350 eggs/day, whereas *S. japonicum* females produce ~2200-3000 eggs/day (Moore and Sandground, 1956; Cheever et al., 1994). One-third to one-half of the eggs either pass through the wall of the intestine and are voided with faeces (*S. mansoni* and *S. japonicum*) or traverse the bladder wall and are excreted in urine (*S. haematobium*). When an egg contacts fresh water, a miracidium hatches and infects any available intermediate host snail to continue the life cycle.

Blood flow also sweeps some *S. mansoni* and *S. japonicum* eggs back to the liver where they become enveloped within granulomatous, immune response-mediated inflammation (Warren, 1964, 1978), from which pathological sequelae of periportal fibrosis, portal hypertension, hepatosplenomegaly and esophageal and gastric varices ensue, constituting the disease intestinal schistosomiasis. *Schistosoma haematobium* egg-induced immunopathology, similarly due to egg-induced granulomatous inflammation, is located in urino-genital tissue.

1.2. Schistosome sexual biology

There is a complex interaction between schistosome male and female worms which involves mate-finding, pairing, migration, the stimulation of female growth and maturation, insemination and the continuous production and release of fertilized eggs. Much has been done to characterize the sexual biology of schistosomes and the topic is the subject of several detailed reviews (Popiel, 1986; Ribeiro-Paes and Rodrigues, 1997; Loverde and Chen, 1991; Kunz, 2001; Hoffmann, 2004; LoVerde et al., 2004; Mone and Boissier, 2004; Steinauer, 2009; Galanti et al., 2012; Lu et al., 2016).

Adult schistosomes become paired before reproductive maturation of the female is complete and they mostly remain paired thereafter. The means by which schistosome male and female worms become paired – for example, through the action of pheromones (Armstrong, 1965) or by thigmotaxis and trial and error (Michaels, 1969) - is not known exactly. Mating among worm pairs separated for up to 72 h in vitro was significantly greater with original than with non-original partners (Shirazian and Schiller, 1982).

It has long been known that the completion of female worm growth and reproductive maturation, and the maintenance of female fecundity, are dependent on a close association between the sexes (Severinghaus, 1928; Moore et al., 1954; Erasmus, 1973). Thus, worms in female-specific single-sex infections do not mature and in the continual absence of male worms they remain stunted. Access of females to the male ventral groove, the gynaecophoric canal, results in mitotic activity (Den Hollander and Erasmus, 1984) and differentiation in the female, leading to a fully developed vitellarium and ovary (Erasmus, 1973; Shaw and Erasmus, 1981). Pairing is also a prerequisite for migration from the hepatic portal vein to the mesenteric veins (Standen, 1953).

The female worm's reproductive system comprises the majority of her body mass and so a large portion of energy supply is devoted to reproduction. The posterior two-thirds of the *S. mansoni* female consists of the vitellarium, a proliferative tissue that produces cells

which surround the ovum and provide the precursor proteins for egg shell formation and nutrients for the developing embryo. *Schistosoma mansoni* females can survive for 30 years or more in the host (Harris et al., 1984), and because the vitelline gland produces approximately 11,000 cells per day (30-40 required per egg) (Erasmus, 1986), a single female worm may produce 1.3×10^8 mature vitellline cells in her lifetime.

In most schistosome species virgin females that inhabit hosts in which there are no male worm counterparts remain considerably smaller than those recovered from mixed infections in which they were paired with males (Erasmus, 1973; Shaw, 1987). Stunted *S. mansoni* female worms have an immature reproductive system and do not produce eggs; the ovary, ootype, and uterus are present in the immature female, but neither the vitellaria nor the Mehlis gland that surrounds the ootype where eggs are formed, are fully developed (Erasmus, 1973; Shaw, 1987). The failure of vitellarial development in the absence of male parasites has been deemed to be largely a consequence of ongoing apoptosis, rather than a lack of cellular proliferation within that organ (Galanti et al., 2012).

The male-derived stimulus is independent of sperm transfer and fertilization (Basch and Basch, 1984) and is not highly species-specific (Jourdane et al., 1995), but intimate association between the male and female worm is necessary and is achieved by the female residing within a ventral groove, the gynaecophoral canal of the male. In a series of noteworthy studies, segments of male worms were transfected with intact immature female worms of the same species into hamsters or maintained in vitro (Popiel and Basch, 1984; Basch, 1988). In both situations vitelline development in the females occurred only in the region of contact with a male segment, and any male segment that could clasp the female worm stimulated development. The uptake of tritiated thymidine was also greater in areas of immature female worms in contact with male segments than in non-contact areas (Basch,

1988). Thus, while the male stimulus is spatially distributed along the gynaecophoral canal, its effect on female vitelline gland development appears to be localized.

Male schistosomes undergo normal morphological development, whether isolated from single-sex or bisexual infections, although some differences between males from single, as opposed to bisexual, infections have been reported (Aronstein and Strand, 1984). Very recently, several genes have been found to be up-regulated in males derived from single-sex male worm infections compared with males from bisexual infections (Haeberlein et al., 2019). Reproductive development in the male seems not significantly influenced by the presence of the female. However, when egg-laying female schistosomes are separated from their male partners and are surgically re-implanted alone into the host, they stop producing eggs and regress reproductively to an immature state similar to that found in females that are unpaired in vivo (Clough, 1981; Popiel et al., 1984). If such regressed females pair again with males, normal reproductive activity resumes, even after months in the regressed state (Clough, 1981). The stimuli for female growth and reproductive development appear to be independent (Armstrong, 1965; Basch and Basch, 1984). In this context, however, an 86 kDa glycoprotein termed gynaecophoral canal protein (SmGCP) has been shown to localize to the surface of the gynaecophoral canal of the male and to the entire surface of the en copula *S. mansoni* female, but was not present on non-mated males or immature females (Aronstein and Strand, 1985; Bostic and Strand, 1996). GCP is involved in promoting intimate contact between male and female worms as small interfering (si)RNA duplexes targeting the *S. japonicum* GCP gene abolished parasite pairing both in vitro and in vivo (Cheng et al., 2009).

The exact mechanisms by which males control female morphological and sexual maturation are unknown, but suggestions include transfer of biomolecules (Armstrong, 1965; Silveira et al., 1986; Ribeiro-Paes and Rodrigues, 1997), physical massaging of the female to assist nutrient uptake and feeding (Gupta and Basch, 1987; Basch, 1990) and/or induction of

a sex-specific signalling cascade (Schussler et al., 1997). Early experiments (Atkinson and Atkinson, 1980; Basch and Nicolas, 1989) putatively demonstrating transfer of proteins from male to female worms deserve to be replicated and enhanced with current mass spectrometric and proteomic tools in order to identify the proteins in question.

1.3. In vitro-cultured schistosomes

Schistosome worms survive well during in vitro culture and they can be grown from the early schistosomulum (somule) to the adult worm stages (Basch, 1981a), but worms grown in vitro from the schistosomula stage produce no viable eggs (Basch, 1981b). Adult egg-producing worm pairs placed in culture cease producing many viable eggs after only a few days incubation (Basch and Humbert, 1981) and the rate of decline in egg production in vitro is greater in unpaired than in paired females (Galanti et al., 2012). Worms that have been perfused as adults from mice, maintained in culture for several weeks and then implanted back into mice are capable of resuming production of viable eggs, although this is not so for implanted worms grown from the larval to the adult stage in vitro, indicating an absence and irreplaceability of some sort of stimulus during early growth in in vitro culture (Basch and Humbert, 1981; Basch and Rhine, 1983). An electron microscopic examination of female worms grown in vitro indicated the structure of the Mehlis gland, ootype, and uterus resembled that of in vivo adult females, but in the ovary the oocytes tended to be degenerate, and within the ootype and uterus the oocytes were not embedded within egg-like material (Irie et al., 1983); it was concluded that a dysfunction of ovarian development was the primary reason for the reproductive failure of schistosome pairs grown in vitro under those experimental conditions.

In an investigation of gene expression in ex vivo female worms, the genes that are expressed in both genders was not affected by contact with the male, but the transcript levels of genes specifically expressed by female worms decreased within a few days following separation from males; re-mating of uncoupled females with males led to the re-initiation of transcription, thus providing evidence for an influence of the male on gene transcription in the female (Grevelding et al., 1997). This influence was not, however, sufficient to sustain egg production by cultured worms.

The loss of vitellarial cellularity associated with regression in vitro is associated with profound apoptotic vitelline cell death, which is not apparent in the vitellaria of paired females immediately ex vivo, and which develops in vitro regardless of whether males are present or not (Galanti et al., 2012). Thus, as Galanti et al. (2012) stated: '…while clearly sufficient to allow and sustain female development in vivo, male parasites are insufficient to prevent vitelline cell apoptosis, normal vitelline cell proliferation, vitellarial atrophy, or female sexual regression in vitro, suggesting that an additional factor(s) present in the host, but absent in our tissue culture conditions, is playing a critical role in female reproductive tract health.'

Considerable effort has been spent on devising in vitro culture conditions that support schistosome development and the subsequent production of mature, viable eggs needed for life-cycle maintenance and research (Cheever and Weller, 1958; Smith et al., 1976; Basch, 1981a,1984; Mann et al., 2010; Wang et al., 2015). A method for culture of schistosomula in a complex medium has been described by Freitas and Pierce at the Schistosomiasis Resource Centre (http://www.afbr-bri.com/schistosomiasis/standard-operating-procedures/culturing-schistosomula/), based upon the work of Basch (Basch, 1981a, 1981b). The culture medium contains human serum, lactalbumin hydrolysate, mouse red blood cells, insulin, thyroid

hormone, hydrocortisone, serotonin and vitamins. However, the authors indicate that in vitro-cultured schistsomula do not grow at the same rate as those in a permissive host, nor do they become fecund adults, although approximately 50% of them mature with fully formed guts, and 10% develop into sexually distinct male and female worms.

A variety of strategies intended to promote in vitro-cultured female schistosome worm maturation and fecundity have been tested, including inclusion in the culture media of extracts of male worms (Shaw et al., 1977; Popiel and Erasmus, 1981), portal blood from a permissive host (Shaker et al., 2011) and transferrin (Clemens and Basch, 1989). A relatively recent publication stresses the likely importance of fatty acids (Pearce and Huang, 2015) and most recently Wang et al., (2019) have recommended supplementing Basch's 'BM169' medium (Basch, 1981a) with ascorbic acid, red blood cells, and cholesterol. None of these strategies has resulted in normal (in vivo) rates of egg production.

There is a consensus that the cytokine transforming growth factor beta (TGF-β) and the signalling pathways it mediates are important for female reproductive development and egg embryogenesis (Freitas et al., 2007; Knobloch et al., 2007; LoVerde et al., 2009; Ting-An and Hong-Xiang, 2009). Is limited access to, or availability of, this cytokine to female worms in single-sex infections and to worms in in vitro cultures the factor which impairs egg production?

2. TGF-β

2.1. TGF-β and schistosomes

TGF-β is one of a large number of related polypeptide growth factors and is produced or released by lymphocytes, monocytes/macrophages and platelets. TGF-β and related cytokines are capable of regulating an array of cellular processes including cell proliferation,

lineage determination, differentiation, motility, adhesion, apoptosis, tissue homeostasis and regeneration (Massague, 2012). Upon activation in mammals (see below) TGF-β homodimers form a complex with two TGF-β type II and two type I receptors, and the inactive type I receptor is phosphorylated by the constitutively active type II receptor (Shi and Massague, 2003). Phosphorylation of a succession of cytoplasmic signal-transducing proteins (Smads and others) follows, resulting in transmission of the signal initiated by TGF-β to the nucleus (Shi and Massague, 2003).

Seven type I receptors and five type II receptors are found in mammals and interactions with contiguous or non-contiguous molecular surfaces determine the specificity of ligand–receptor pairings (Moustakas and Heldin, 2009). TGF-β binds exclusively to a particular type II receptor, while other TGF-β family proteins such as activin and bone morphogenetic protein (BMP) (see below) react with type II receptors different from that used by TGF-β. Type I receptors are also known as activin receptor-like kinases (ALKs), a nomenclature used to deal with the problem of one ligand signalling via many receptors, or many ligands signalling via the same receptor (Shi and Massague, 2003; Moustakas and Heldin, 2009; Massague, 2012).

An early indication that TGF-β may be involved in schistosome physiology was the finding of a divergent member of the type I TGF-β family of cell-surface receptors (SmTβRI or SmRK-1) expressed on the dorsal surface of the male worm tegument (but not the female), and up-regulation of expression of receptor-coding messenger RNA and protein in the parasite following infection of the mammalian host (Davies et al., 1998). This was followed by discovery of an orthologous type II TGF-β receptor (SmTβRII) on both male and female worm surfaces, including the gynaecophoral canal surface and some internal tissues of the female (Forrester et al., 2004).

Recent annotation of *S. mansoni* protein kinases (Grevelding et al., 2018) and subsequent Basic Local Alignment Search Tool protein (BLASTp) searching of WormBase Parasite (https://parasite.wormbase.org/index.html) by the current authors identify five putative TGF-β receptors (Smp_049760, Smp_093540, Smp_124450, Smp_144390, and Smp_334370) with representatives of both classes evident.

TGF-β itself has not been detected in *S. mansoni*, but two TGF-β-like ligands have been found: a bone morphogenetic protein-like molecule (SmBMP) which is found particularly in male worm protonephridia (Freitas et al., 2009) and an Inhibin/Activin-like molecule (SmInACT), (Freitas et al., 2007). Based on real-time reverse transcription (RT)-PCR analyses, SmInAct is abundantly expressed in ovipositing females and the eggs they produce, and its expression is tightly linked to the reproductive potential of the worms (Freitas et al., 2007). The receptors for which SmBMP and SmInACT are the ligands, their location in the parasite and possible roles for the two cytokines additional to involvement in reproduction have not yet been described, although Freitas et al. (2007, 2009) speculate that both SmInAct and SmBMP could have an effect on host cells.

Other major components of a TGF-β signalling pathway have been identified in schistosomes and evidence obtained for their expression in the parasite's reproductive organs (Beall et al., 2000; Knobloch et al., 2007; Loverde et al., 2007; LoVerde et al., 2009; Beckmann et al., 2010). Furthermore, in vitro results that are consistent with host-derived TGF-β being involved in schistosome physiology have been obtained. Thus, human TGF-β can influence gene expression in adult *S. mansoni* (Oliveira et al., 2012), although in a study which did not consider whether there was an effect on egg production. Perhaps of particular interest, TGF-β up-regulated expression of GCP (SmGCP), which was in turn suppressed by RNA interference (RNAi)-mediated silencing of SmTβRII (Osman et al., 2006). SmGCP was found to exhibit an expression peak at 28 days p.i. (Osman et al., 2006), which coincides with

the onset of worm mating. In light of the observation of extensive apoptotic vitelline cell death in reproductively impaired female worms (Galanti et al., 2012), it is noteworthy that TGF-β has an anti-apoptotic effect (Schuster and Krieglstein, 2002). It is of interest that Sm*fst* is one of the genes up-regulated in *S.* mansoni males from single sex infections when compared with males from worm pairs (Haeberlein et al., 2019). Sm*fst* is a follistatin orthologue and follistatins are known to be negative regulators of TGF-beta signalling pathways and thus negatively influence TGF-induced signal transduction. Since TGF-beta signalling is likely involved in reproductive processes in schistosomes Sm*fst* might prevent the activation of a TGF-beta-induced signalling pathway in male worms prior to their pairing with female worms (Leutner et al., 2013).

Many details of the molecular reactions between TGF-β-like cytokines and their receptors in schistosomes have still to be elucidated. There is nevertheless evidence that host TGF-β initiates signal transduction through a schistosome TGF-β signalling pathway. Thus, Beall and Pearce (2001) showed that a chimeric receptor containing the extracellular domain of SmTβRI joined to the intracellular domain of human type I TGF-β receptor bound radiolabeled TGF-β and could activate a luciferase reporter gene in response to both TGF-βI and TGF-β3. Confirmatory results were obtained using full-length SmTβRI. They concluded these experiments implicated TGF-β as a ligand for SmTβRI and as a potential host-derived regulator of parasite growth and development (Beall and Pearce, 2001). In addition, in vitro and in vivo studies have shown that human TGF-β1 binds to SmTβRII, which forms a complex with SmTβRI and activates it by phosphorylation. SmTβRI then interacts with and phosphorylates SmSmad2, dissociating the latter from the receptor complex and allowing formation of a hetero-oligomeric complex with SmSmad4 and activation of a reporter construct or upregulation of transcription in a target gene (summarized in LoVerde et al. (2007, 2009)). However, as stated by those authors, occurrence of these events during

infection ‘is yet to be demonstrated’. There is also the question whether a signal initiated by host-derived TGF-β on the worm surface (male or female) can migrate through multiple tissues and directly affect vitellocytes, raising the further question whether as yet unknown schistosome cytokine orthologues may activate the TGF-β signalling pathway in reproductive tissues (Knobloch et al., 2007). Furthermore, the possibility that parasite-derived ligands of the schistosome TGF-β receptors are active in vivo, but not in vitro, should be considered.

While there is a consensus that TGF-β seems to be an important component in development and maintenance of schistosome reproductive capacity and that the host may be the source of the cytokine, alignment of the *S. mansoni* type I and type II receptor sequences with those of other type I and II receptors indicated they share little conservation at the sites which structural studies of mammalian growth factor-receptor complexes have identified as contacting the growth factor (Loverde et al., 2007). These differences raise the possibility that although *S. mansoni* receptors may well interact with host TGF-β, it may be in a manner that is entirely distinctive compared with the host TGF-β receptors (Loverde et al., 2007) or, indeed, that in vivo there is no such interaction between the respective host and parasite elements; i.e., the interactions in the above-mentioned in vitro studies might be purely fortuitous, and not indicative of any biologically relevant interaction in vivo.

Due to production of TGF-β in latent form, the existence of numerous extracellular modulators that can control its potent bioactivities (Chang, 2016) and the probable rapid clearance of the free form, only a small amount of biologically active cytokine is likely to be available in host plasma (Khan et al., 2012). This may be insufficient for the worms’ needs and the question is: by what means might schistosomes obtain necessary amounts of the cytokine?

2.2. How might schistosomes acquire host TGF-β?

2.2.1. From a latency-forming complex of proteins?

Most TGF-β is produced in a complex with other proteins that confer latency. If the parasite uses host-derived TGF-β it will likely somehow have to release it from its latent-complexed form. Little work has been done to investigate this question.

TGF-β is a ~50 kDa homodimer synthesized with a pro-peptide. The cytokine dimer is cleaved from its pro-peptide, but after this cleavage TGF-β and a latency-conferring, associated peptide (LAP) remain strongly associated via non-covalent interactions. This small latent complex (SLC) is linked to a single latent TGF-β-binding protein (LTBP) by a pair of disulphide bonds between LTBP and LAP. The tripartite complex of TGF-β, LAP and LTBP is called the large latent complex (LLC), which remains bound to the extracellular matrix (ECM) via a hinge domain of the LTBP, thus anchoring TGF-β but rendering it potentially available to cells. Structural studies have shown that TGF-β adopts a similar structure in the free, latent, and receptor bound states, but when bound to LAP the TGF-β receptor-binding sites are shielded by regions of the propeptide (reviewed in Lawrence (2001); Worthington et al. (2011); Robertson and Rifkin (2016)).

In order for TGF-β signalling to occur, therefore, the growth factor must be released from the latent complex bound to the ECM, a process referred to as ‘activation’. Both of the main mechanisms implicated in TGF-β release/activation involve integrins, dimeric transmembrane receptors comprising alpha and beta peptide subunits that mediate cell adhesion (Hynes, 2002). Thus, tethering of the ECM-bound latent complex to cell surface-bound integrins via an Arg-Gly-Asp (RGD) motif on the SLC: (i) induces, by traction, a conformational change in the latent complex that results in release of active growth factor; and/or (ii) renders the latent complex accessible to a cell surface protease such as membrane-

type metalloproteinase 1, which cleaves LAP and releases TGF-β. Integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$ and $\alpha_5\beta_8$ have been implicated in this process (Lawrence, 2001; Worthington et al., 2011; Robertson and Rifkin, 2016).

Schistosoma mansoni does synthesize integrin homologues; four α-integrins (Smα-Int1-4) and one beta-integrin (Sm-βInt1) were cloned and characterized by molecular and biochemical analyses and Smβ-Int1 interacted and co-localized in the reproductive organs with known schistosome cellular tyrosine kinases (Beckmann et al., 2012). Both Smα- and Smβ-integrins were also detected in exosome-like vesicles derived from *S. mansoni* and have been found on the surface of the parasite (Samoil et al., 2018), but whether the schistosome integrins can interact with LAP, as described for interaction of mammalian integrins with RGD-containing proteins (Xiong et al., 2002; Xiao et al., 2004), has not been investigated. The same applies to metalloprotease activity which has also been implicated in the release of TGF-β from its latent forms (Yu and Stamenkovic, 2000).

It may also be noted that several additional factors, named 'variables' by Massague, (2012), outside the target cell can affect the extent of stimulation by a TGF-β cytokine. These include ligand traps, antagonistic ligands and accessory receptors (Massague, 2012), the existence of which has not yet been demonstrated in schistosomes.

2.2.2. From alpha2-macroglobulin (A2M)?

Alpha 2-macroglobulin (A2M) is a host protein known to interact with TGF-β and is also associated with *S. mansoni*; might it be involved in providing host-derived TGF-β to the parasite?

A2M is a member of an ancient protein family and considered by some to be a component of the innate immune system (Armstrong and Quigley, 1999; Rehman et al.,

2013). It is highly conserved in animal species separated by over half a billion years of evolution. The mammalian form is a major blood glycoprotein, constituting 5-10% plasma protein content. It is synthesized principally by the liver, but also by other cell types and assembled from four identical 180 kDa subunits into a 720 kDa tetramer (Marrero et al., 2012).

The best-known property of A2M is its ability to bind a broad spectrum of endoproteases of all four mechanistic classes, which it accomplishes using a unique trapping method and to facilitate their clearance. Following interaction with a protease, A2M undergoes limited proteolysis at its so-called 'bait region', leading to a large irreversible conformational change, physically containing the protease molecule(s) within the cage-like structure and yielding an 'activated' or electrophoretically 'fast' form (A2M*) (Barrett and Starkey, 1973; Barrett et al., 1979; Salvesen et al., 1981; Feldman et al., 1985; Sottrup-Jensen, 1989; see also Figure 1C).

As a result of interaction with an endoprotease, the receptor recognition site for the low-density lipoprotein receptor-related protein (LRP) of A2M* becomes exposed (Strickland et al., 1990; Herz and Strickland, 2001). In vivo, the A2M*/protease complex is rapidly cleared by LRP-mediated endocytosis and subsequently degraded (Imber and Pizzo, 1981), so it typically represents less than 1% of the total A2M in blood plasma of adults (Banks et al., 1990). The trapped protease molecules become covalently linked to the A2M as a result of cleavage of a thioester bond in the cavity, but their enzymatic activity is retained at least with respect to small-sized molecules such as chromogenic substrates that can pass through the arms of the proteinase-trapping cage (Barrett and Starkey, 1973; see also Fig. 1C).

A2M has also been shown to bind to, and thus facilitate the clearance of, a diverse range of non-covalently bound cytokines and growth factors (Borth, 1992) including TGF-β

(O'Connor-McCourt and Wakefield, 1987; LaMarre et al., 1991a; Wollenberg et al., 1991; Hall et al., 1992; Feige et al., 1996; Webb et al., 2000). The cytokines associate with A2M*, i.e., after it has been activated by a protease, but generally not with native A2M (LaMarre et al., 1991a, 1991b). It has been suggested that the complexing of TGF-β with A2M is a means of conferring latency on the cytokine (Huang et al., 1988). It may also be a mechanism that allows targeting of a biologically active peptide to different cell types expressing the A2M receptor and after dissociation to affect the process of intracellular ligand sorting, thereby modulating cell function (Borth, 1992; Stouffer et al., 1993).

In mammals A2M* interacts with another signal-transducing protein, the 78 kDa glucose regulated protein (GRP) 78, (Misra et al., 1994, 2002; Pizzo, 2015), also known as the binding immunoglobulin protein BiP and heat shock protein HSPA5 (HSP70 family protein 5). Only a small amount of GRP 78 is found on the surface of macrophages (unlike LRP), but it is in a "co-receptor" relationship with LRP on the surface of prostate cancer cells and essential for A2M*-induced signal transduction in those cells (Misra et al., 2002). As noted above, extensive apoptotic vitelline cell death occurs in reproductively-impaired female worms (Galanti et al., 2012) and in this connection it is of interest that GRP 78 has an anti-apoptotic effect, albeit particularly in cancer cells (Lee, 2014).

3. Schistosomes and A2M

3.1. An association between S. mansoni and A2M

It is tempting to speculate that A2M* with bound TGF-β could be a means by which schistosomes obtain the host cytokine, as mouse A2M antigenic determinants have been found on *S. mansoni* adult worms taken from mice (Damian et al., 1973; Gearner and Kemp, 1994). In one report, evidence for mouse A2M was surprisingly found even on worms from rhesus monkeys, as well as on those from mice (Kemp et al., 1976). Further evidence for an

association between A2M and *S. mansoni* worms in mice is given in Figs. 1A, B and D. It may not be just coincidence that male worm tubercles are where both anti-A2M antibodies (Fig. 1D) and TGF-β receptors (Davies et al., 1998; Forrester et al., 2004) are found.

At the time of the earlier observations, the most likely explanation for the association between A2M and schistosomes was that the latter was an immune evasion mechanism; i.e., a function similar to that served by host blood group antigens (Smithers et al., 1969), immunoglobulins (Kemp et al., 1977) and/or the serine protease inhibitor contrapsin (Modha and Doenhoff, 1994). The relationship between *S. mansoni* and A2M was, however, investigated further and it was shown that: (i) mice with patent *S. mansoni* infections had lower concentrations of A2M in their serum than uninfected control mice (Fig. 1E); and (ii) in immunoelectrophoresis immunoprecipitin arcs of A2M from the serum of patently infected mice migrated faster towards the anode than the arcs from uninfected mice; i.e., some of the A2M in the infected mice was in activated A2M* form (Fig. 1F). This latter finding is unusual; as already mentioned activated A2M* is rapidly removed from circulation (Imber and Pizzo, 1981) and it constitutes only a small fraction of the A2M in blood (Banks et al., 1990). How A2M has apparently here been activated in vivo remains to be elucidated. Additional evidence that schistosomes influence turnover of A2M is indicated by a recent comparison of the proteomes of human serum that had been incubated in vitro with or without live adult *S. mansoni* worms; the serum sample that had been incubated with worms was found to have ~50% less A2M than that incubated without worms (Da'dara et al., 2017).

If A2M does fulfil the TGF-β delivery role suspected here, it might be further speculated that under some circumstances the availability of the host macroglobulin might become limited. This may explain why the fecundity of *S. mansoni* infections with high male:female worm ratios is reduced in both mice (Harrison et al., 1982) and baboons (Damian and Chapman, 1983); i.e., possibly because the excessive number of male worms

are usurping the supply of A2M and thus denying females' needs. This explanation does not extend to expression of SmGCP, however, as that protein is undetected or severely diminished in unpaired male worms (Bostic and Strand, 1996).

3.2. Orthologous genes

Adult schistosomes and schistosomula have been found to possess tegument surface membrane receptors that have the capacity to bind lipoproteins (Rogers et al., 1990). A BLASTp search using human low density lipoprotein receptor-related protein 1 (LRP1; Uniprot ID: Q07954; 4544 amino acids (a.a.); 505 kDa) as query against the current version of the *S. mansoni* genome (www.wormbase.parasite.org) revealed Smp_346610.3 (also known as Smp_179370), a ~266 kDa protein (2371 a.a.) which when analysed using InterPro (www.ebi.ac.uk/interpro) possessed domain architecture characteristic of LDL receptor class A proteins. There are four putative splice variants of the Smp_346610 gene, with Smp_346610.3 being the largest. Furthermore, the previously annotated Smp_179370 is expressed in cercariae, schistosomula and adult worms by RNAseq (GeneDB; www.geneDB.org). Thus machinery for the potential capture and endocytosis of A2M* associated with TGF- β may be present and expressed in schistosomes, although the location of the products of these genes, if such exist, in or on the worms is not known. Mammalian LRP molecules are accompanied by a 39 kDa receptor-associated protein (RAP) which serves as a molecular chaperone to assist the folding of LDL-receptor family proteins and their passage through the secretory pathway (Bu and Schwartz, 1998). However, we found no evidence for an orthologue of the RAP gene in the *S. mansoni* genome.

A gene that is an orthologue of GRP 78, the other receptor for A2M*, has been found in *S. japonicum* (Scott and McManus, 1999) and a BLASTp search of the *S. mansoni* genome at WormBase ParaSite using human GRP78 (Uniprot ID: P22010; 679 a.a; ~74 kDa) as input

sequence revealed Smp_049550, a ~71 kDa (648 a.a.) HSP70-like protein with high (~75%) sequence consensus to the human protein.

Schistosoma mansoni has two genes with some homology to mammalian A2M: Smp_133880 annotated 'Putative alpha-2-macroglobulin' and Smp_089670 annotated 'Putative macroglobulin/complement' (Protasio et al., 2012). The latter protein, described by (Castro-Borges et al., 2011) as an A2M, was present in worm vomitus. The sequences of these two proteins have little homology with each other or with that of mouse A2M, and an analysis of whether the schistosome macroglobulins have peptide-binding properties has not been done. Their function is also not yet known.

3.3. TNF-α and amyloid beta peptide (Aβ) also bind to A2M

Interestingly, TNF-α also binds non-covalently to A2M* (Wollenberg et al., 1991), but unlike TGF-β this cytokine is not synthesized in latent form. A putative homologue of the TNF-α receptor (TNFR) has been found in *S. mansoni* and treatment of adult worms with human TNF-α caused a significantly altered expression of 1857 genes (Oliveira et al., 2009). Amiri et al. (1992) showed TNF-α to be required for *S. mansoni* to produce eggs and for the eggs to be excreted in mice with severe combined immunodeficiency (SCID). Cheever et al. (1999), however, found little evidence that TNF-α alone could reconstitute early fecundity in such mice. A lack of this cytokine did nevertheless cause abnormal accumulation of worms in the livers of TNF-/- mice with 6 week-old infections (Davies et al., 2004).

A2M has at least three domains with distinct functions. The 'growth factor binding' domain is located mid-molecule close to the protease 'bait region', while the LRP receptor recognition sequence that enables A2M* to be endocytosed is close to the C terminus (Gonias et al., 2000; Mettenburg et al., 2002). The A2M domain that is reactive with TGF-β targets the receptor-binding sequence of the cytokine (Arandjelovic et al., 2003) and is located

within the central cavity of the protease inhibitor (Webb et al., 2000), incidentally posing the question of how the cytokine is released (as there is little if anything in published literature to indicate how endocytosed A2M* is metabolised.

It is of potential interest that A2M also contains a sequence which enables it to bind to amyloid-beta peptide (Aβ) (Hughes et al., 1998), a property that possibly connects schistosomes and A2M with Alzheimer's disease (AD). The Aβ recognition sequence is, however, close to the LRP recognition site on A2M and therefore distinct from the growth factor binding site (Mettenburg et al., 2002).

Aβ peptides are generated by successive proteolytic cleavages of the amyloid precursor protein (APP) and they accumulate as large insoluble aggregates (plaques) in AD brains, constituting a major pathological feature of this disease. It is believed that brain and peripheral soluble Aβ are in equilibrium and it has been hypothesized that a reduction in peripheral Aβ can lower its concentration in the brain, thereby reducing formation of the plaques of aggregated Aβ - the so-called 'peripheral sink' hypothesis (Karran et al., 2011). Association of Aβ with A2M and degradation of the complex could be one means by which toxic accumulation of the peptide is limited. The peripheral sink hypothesis is, however, the subject of controversy (Makin, 2018) and many clinical trials aimed at reducing amyloid plaque and/or Aβ have failed, perhaps because the drugs are administered too late after the development of symptoms (Herrup, 2015); see also for example: https://www.alzforum.org/news/research-news/biogeneisai-halt-phase-3-aducanumab-trials.

Unlike the interaction with TGF-β, Aβ associates equally well with both activated and native forms of A2M (Du et al., 1997), but nevertheless any mechanism by which schistosomes might be involved in metabolism of Aβ would require the A2M carrying the peptide to have been activated, e.g., by a proteinase.

4. Conclusions: testing the hypothesis and importance of the question

This is a potential solution to an interesting biological question, of interest and relevance not only to those who work with schistosomes, but possibly also with respect to other helminth species which are unable to reproduce in vitro.

As outlined above, there is a consensus that schistosomes need a host factor, likely TGF-β, to stimulate development of female worm reproductive tissues and production of eggs, and to induce synthesis of the GCP. However, so far little thought seems to have been given to the actual means by which schistosomes obtain the TGF-β they are deemed to need. In vivo studies have provided scant evidence that a biologically active form of the cytokine acts directly on the worms, and in any case, as already argued it is probable that only very small amounts of free TGF-β are available in host blood. While human TGF-β has an effect on gene expression in worms in vitro (Osman et al., 2006; Oliveira et al., 2012), there are no reports that such in vitro exposure to the cytokine has any remedial effect on impaired female worm reproduction rates.

Two possible sources of TGF-β have been described above; from the large latent complexes in which it is bound and/or from that borne by alpha 2-macroglobulin or GRP 78. Experiments could be performed to investigate whether schistosome worms possess factors such as surface-bound integrins that can interact with the TGF-β-bearing latency complexes to release the cytokine, with or without mediation of a (metallo)protease.

Alternatively, it could be tested whether A2M that has reacted with a protease or been treated with an ammonium salt or methylamine (Barrett et al., 1979) and been ‘spiked’ with TGF-β prolongs egg production by ex vivo adult worms placed in culture. The relative concentrations of protease and A2M are important as A2M molecules complexed with one proteinase molecule bind TGF-β, while complexes containing two enzyme molecules do not (Hall et al., 1992). The apparent effect of schistosome infection on A2M metabolism in vivo,

as illustrated in Fig. 1E and F, needs further investigation due to its potential relevance to this hypothesis. The intricacies of how a host-derived factor taken up by a male worm, such as TGF-β, or a signal that it generates, finds its way to female reproductive tissue may be difficult to elucidate, but further investigations on where schistosome orthologues of low density lipoprotein receptors and GRP 78 are localized would seem worthwhile.

The ideas expressed above and the role ascribed to A2M in particular are speculative and the reasons for the association of A2M with *S. mansoni* worms remain unknown. As suggested for other host-derived molecules on schistosomes, it may 'simply' be a passively acting immune-evasion device, although being an inhibitor of all classes of endoprotease it may help protect the worm against host blood coagulation and/or immune system proteolytic activity. Even if the hypothesis bears some resemblance to reality, many questions remain: for example, is TGF-β transferred directly to the worm(s) from the complex proteinaceous shell bestowing latency upon it, or via one or both of the two molecular cytokine acceptor devices A2M* and GRP 78. An investigation into the means by which A2M becomes attached to or incorporated by the worm may contribute to better understanding, as may an examination of the host/parasite relationship of *S. mansoni* in A2M gene-knockout mice (Umans et al., 1995).

Currently many laboratories working on schistosomiasis run their own complete schistosome life-cycles requiring successive passages through laboratory rodent definitive hosts (mostly mice and hamsters) and intermediate host snails. Purchase and maintenance of laboratory animals is expensive and their use is fraught with potential difficulty due to strict regulation of vivisection in many countries. A method that enables cultured worms to produce mature, hatchable eggs in vitro would abrogate the need for definitive hosts. Such a method applied to *S. haematobium* worms would be particularly advantageous as the life cycle of this species is more difficult to maintain in the laboratory than that of *S. mansoni*.

Schistosome eggs and molecules derived therefrom are valuable for diagnosing human schistosomiasis (Hamilton et al., 1998) and they are also interesting and valuable research tools, particularly with respect to their immunomodulatory properties (Schramm and Haas, 2010; Obieglo et al., 2018). A method of producing mature viable eggs from cultured worms would again bypass the expense and inconvenience of having to exploit laboratory animals for diagnostic and research purposes.

There are extraordinary possibilities if the suggested connection with AD can be supported by evidence. Parenthetically of potential relevance, but with no previous direct connection to the ideas expressed here, a clinical trial of infecting volunteers with single sex male *S. mansoni* cercariae is currently underway: https://clinicaltrials.gov/ct2/show/NCT02755324.

Acknowledgements

The authors received no funding that specifically supported this work. The authors have no conflict of interest.

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Figure legend

Fig. 1. Relationship between mouse alpha 2-macroglobulin and *Schistosoma mansoni.* (A, B) Preparation of rabbit antiserum specific for an anodally-migrating arc in normal mouse serum recognized by rabbit anti-*S. mansoni* adult worm homogenate (AWA). (A) Immunoelectrophoresis; antigen wells, 5 µl of normal mouse serum. Antiserum troughs, 250 µl of: 1, rabbit antiserum against unfractionated AWA; 2, serum from rabbit immunized with anodally-migrating antigen in mouse serum precipitated by anti-AWA (arrow); 3, commercial anti-mouse alpha 2-macroglobulin (A2M); 4, rabbit anti-normal mouse serum. (B) Ouchterlony double immunodiffusion demonstrating reactivity of sheep anti-mouse A2M antibodies with antiserum: 1, rabbit anti-AWA; 2, commercial rabbit anti-mouse A2M; 3, serum from rabbit immunized with anodally-migrating arc precipitated by rabbit anti-AWA; 4, serum in well 3 diluted 1:4 with isotonic saline. Centre well contained 5 µl of normal mouse serum, outer wells 5 µl of fluid. (C) Activation of mouse A2M by chymotrypsin. (a) Immunoelectrophoresis of rabbit anti-mouse A2M antiserum (troughs, 250 µl of antiserum) reacting against normal mouse serum or normal mouse serum incubated with chymotrypsin. Antigen wells contained: 1, (5 µl) mouse serum; 2 (10 µl), mouse serum incubated with chymotrypsin. (b) Similar to (a) but after rinsing in saline to remove non-precipitated material, followed by incubation in a chromogenic chymotrypsin substrate. (D) Representative immunofluorescence of adult *S. mansoni* male worm surface. Worms were incubated in the lab-prepared rabbit anti-mouse A2M antiserum, followed by fluorescein-tagged sheep anti-rabbit IgG. (E) Single radial immunodiffusion of immunoprecipitated A2M in serum samples from *S. mansoni*-infected mice (squares) and uninfected controls (circles). Agar films containing 1 ml of laboratory-prepared rabbit anti-mouse A2M antiserum were reacted with 5 µl of serum samples from infected and control mice; mean diameters of

immunoprecipitin rings (5 mice +/- 1 S.D.) are shown. (F) Immunoelectrophoretic detection of A2M in sera of *S. mansoni*-infected mice. Samples were taken from four mice (lanes 1, 3, 5 and 7) infected with cercariae 44 days previously, and from three age- and sex-matched uninfected controls (lanes 2, 4 and 6). Mouse serum (5 µl) was placed in each well and troughs were filled with 250 µl of rabbit anti-mouse A2M antiserum. Detailed methods can be found in Supplementary Methods S1.

Supplementary information: Materials and methods for results in Figure 1.

1. Experimental animals and parasite

Random-bred, Tyler's Original (TO) strain mice (from Tuck & Sons, Battlebridge, England) were used for laboratory maintenance of the S. mansoni life cycle and for acquisition of schistosome worm antigens. New Zealand White rabbits (Hop Rabbits, Canterbury, England) were used for preparation of antisera against S. mansoni antigens and against mouse serum and its constituents. A Puerto Rican isolate of S. mansoni was maintained by continuous passage in mice, which were infected with cercariae percutaneously as described by (Smithers and Terry, 1965), adapted as in (Doenhoff et al., 1978). Maintenance of the parasite life cycle and other experiments with laboratory animals were performed with strict adherence to the regulations set out in the UK Animals (Scientific Procedures) Act, 1986. Animals were killed by administration of a lethal dose of pentobarbitone anaesthetic. CBA/H-T6T6 mice, infected with S. mansoni as above, were used to obtain serum samples from infected animals and for samples of normal mouse serum from uninfected age- and sex-matched controls.

2. Antigens, antisera and immunological techniques

Adult mixed-sex S. mansoni worms, freshly perfused from heavily infected mice via their incised hepatic portal veins, were homogenized and the homogenates centrifuged as described previously (Doenhoff et al., 1981). Rabbit antisera were raised against the worm antigens and normal mouse serum using previously published approaches (Darani and Doenhoff, 2008). A monospecific rabbit antiserum reactive against a mouse serum molecule that had been immunoprecipitated by immunoelectrophoresis in agar was raised by a method described by (Goudie et al., 1966), adapted as in (Dunne et al., 1986). A sheep antiserum specific for mouse A2M was purchased from R&D Systems (Abingdon, UK).

Immunoelectrophoresis was performed on 84 mm square glass plates carrying 10 ml of 1.5% Difco Noble agar dissolved in 0.075M barbitone buffer (pH 8.6) by previously described methods (Williams and Grabar, 1955, Wieme, 1959), adapted as in (Dunne et al., 1986). Electrophoresis was performed for 2.5 h with a potential difference of approximately 5 v/cm and current of 30 mA/plate.

Single radial immunodiffusion (SRID) was performed as described in (Mancini et al., 1965) adapted as in (Darani and Doenhoff, 2008). Films on 84 mm square glass plates were prepared containing 0.5 ml rabbit anti-mouse A2M antiserum in 12.5 ml of 1.5% agar. 5 µl samples of serum from infected or control mice were placed in separate wells in the agar film. Plates were incubated at 37oC for 24 h and diameters of immunoprecipitin rings measured on images photographed over direct light. Double immunodiffusion (DID) was performed as described previously (Ouchterlony, 1958). SRID and DID were performed with glass plates, agar and buffer as for immunonelectrophoresis.

In order to activate A2M with a proteolytic enzyme and produce the ‘fast’-migrating form (Barrett et al., 1979), normal mouse serum was incubated with bovine pancreatic chymotrypsin (Sigma-Aldrich, Poole, UK; 1 mg enzyme / 1 ml of serum). Proteinase activity

was detected in immunoprecipitated antigen in agar (Uriel, 1963, Barrett and Starkey, 1973) by washing the agar film in excess isotonic saline for 24 h, followed by immersion in a chromogenic substrate mixture consisting of 5 mg N acetyl-DL-phenylalanine β-naphthyl ester (NAPBNE), a substrate suitable for detection of chymotrypsin-like enzymes, and 10 mg Fast Blue B (FBB, a coupling agent) dissolved in 2 ml dimethyl formamide. The substrate mixture was added to 40 ml of 0.5 M phosphate-buffered saline (PBS), pH 7.6, within which solution the agar film carrying immunoprecipitate was incubated for approximately 30 min or until chromogenic staining (pink colouration) was evident. All chemicals were purchased from Sigma-Aldrich.

For immunofluorescence, freshly-perfused adult worms were rinsed in Medium 199 at 37oC, incubated in vitro for 30 min at 37oC with a 1:40 dilution of a rabbit antiserum specific for mouse A2M, rinsed in fresh medium and incubated for 1 h with a 1:200 dilution of flouresceinated goat anti-rabbit IgG (Serotec, Oxford, UK).

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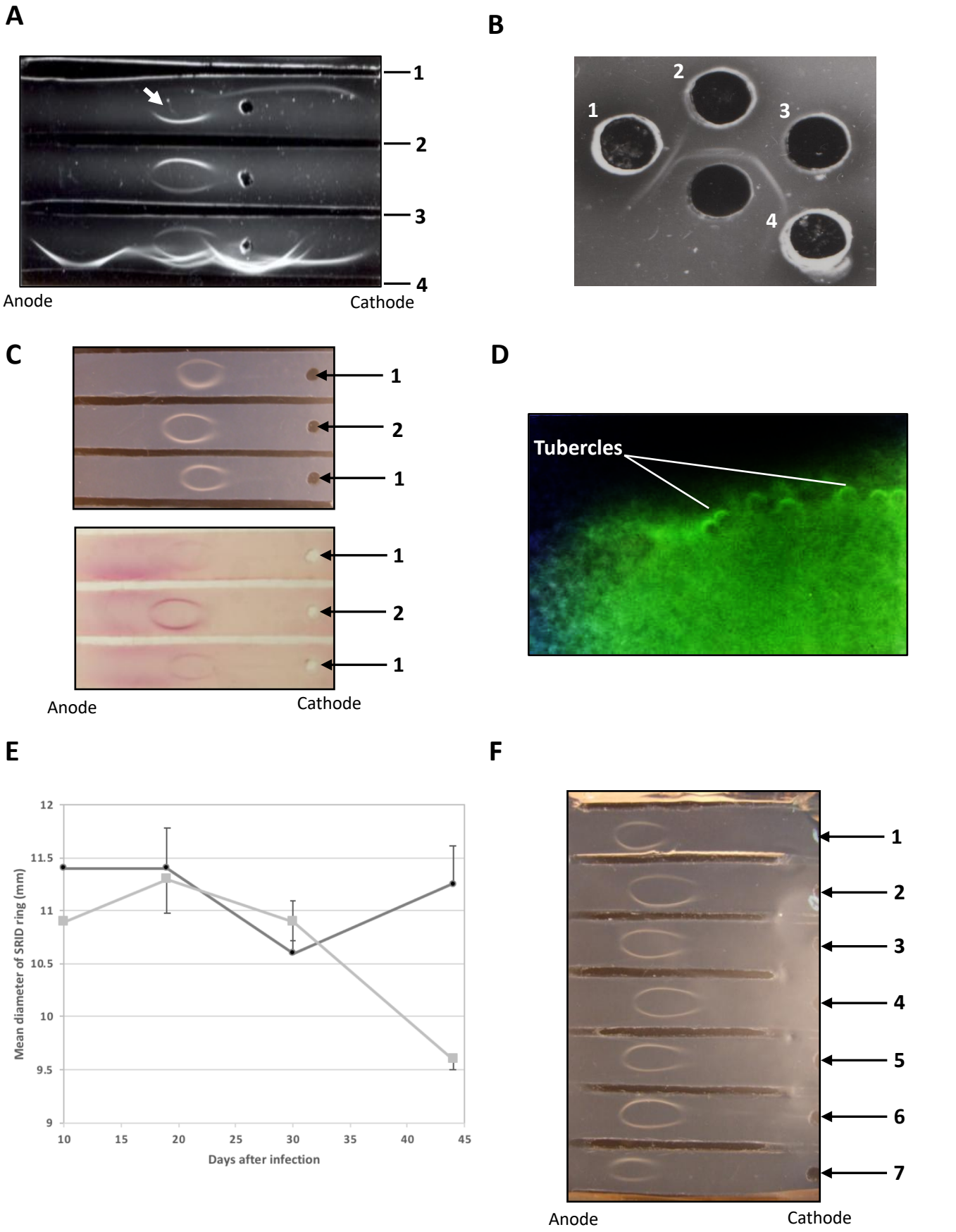
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Figure 1