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Current Opinion in Microbiology

Fasciola hepatica, TGF-β and host mimicry: the enemy within

Helminths parasites often under developmental changes and migration within their definitive host, in addition to establishing chronic infection. Essential to this is the evasion of host immune responses; the canonical Th2 response is effective at clearing parasites resident in the intestine. Conversely, helminths also promote the development of antigen-specific anergy and regulation. This often limits pathology but allows parasite survival, parasite effectors mediating this are the subject of intense study. They may be useful as future vaccine targets or xenogenic therapeutics. *Fasciola hepatica* possesses a family of TGF-like molecules of which one member, FhTLM, is capable of promoting intrinsic and extrinsic effects. Here we review the extrinsic effects of FhTLM on the host macrophage and its consequences for protective immunity. This review also discusses the specificities of FhTLM in light a very recent description of a nematode TGF-β mimic and the effects of endogenous TGF-β.

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Fasciola hepatica

Fasciola hepatica a common trematode parasite with a global distribution causing massive economic losses and animal health problems in livestock, it is also a zoonotic infection and has been reclassified as a re-emerging neglected tropical disease by WHO [1]. *F. hepatica* has an indirect lifecycle, emerging from eggs on pasture to infect a snail intermediate host and undergoing clonal replication [2]. Cercariae emerge from the snail and transform to infectious metacercariae on pasture, when ingested by mammalian hosts and juvenile parasite emerge within the intestine. Control is via the routine application of triclabendazole targeting both the newly excysted juvenile (NEJ) and the adult forms. This is particularly important in livestock where the NEJ can cause acute mortality when present in high numbers. Consistent use in livestock systems has led to the emergency of drug resistance and efforts are underway to isolate the genomic loci/locus responsible [3,4]; these efforts began with the sequencing of the genome which has afforded us the opportunities to identify new effector proteins within *F. hepatica*.

Immune regulation in *F. hepatica*

In its mammalian hosts *F. hepatica* infection induces strong Th2 immune responses [5–8]. This response is characterised by eosinophilia, alternatively activated macrophages, and elevated IgG1, interleukin (IL)-4 IL-5, and IL-13 production [6,9,10,11•]. *F. hepatica* often results in chronic infection with the parasite surviving for prolonged periods of time in the host despite the magnitude of the immune response mounted by the host. For the host to mount protective immunity a dominant Th1 response or a balance of Th1/Th2 responses is essential [12,13]. Th1 responses are down modulated during infection [14,15]. In support of this little to no IFN-γ is detected in bulk PBMCs or CD4 T cells, indeed any produced is transient and rapidly disappears [15].

As chronic infection becomes established, there is a dominance of regulatory environment characterised by suppression of parasite-specific Th1 and Th2 responses and induction of immuno-suppressive cytokines; IL-10 and transforming growth factor (TGF)-β [12,16,17]. Infection of mice with *F. hepatica* recruits macrophages and DCs both expressing high levels of IL-10 [17]. CD4 T cells expresses IL-10 while production of antigenic-specific IL-4 and IFN-γ are suppressed, with suppression of IL-4 and IFN-γ abrogated in IL-10 deficient mice. Moreover, *in vivo* secretion of TGF-β attenuated development of auto-immune disease via suppression of auto-antigen specific IFN-γ and IL-17 production [17]. In ruminant hosts, *in vitro* neutralization of IL-10 and TGF-β in PBMCs isolated from *F. hepatica* infected cattle resulted in increased production of IFN-γ and IL-4 respectively [12]. As a further development of these there is a strong degree of anergy induced in bovine CD4 T-cells that is dependent on the PD-1/PD-L1 pathway and utilising IL-2 regulation in combination with IL-10 and TGF-β secretion. Murine models of disease have provided multiple examples of the PD-1/PD-L1 pathways importance in *F. hepatica*. Injection of *F. hepatica* extract causes upregulation of PD-L2 on peritoneal macrophages

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[18]. PD-L2 knock out mice (KO) mice however demonstrate exacerbated liver pathology and increase susceptibility to infection with high production of IFN-γ and reduced IL-4 and IL-10 production [19]. PD-L2-positive murine macrophages co-cultured with naïve CD4 T cell, caused loss of T-cell function. Cell failed to proliferate or produce IFN-γ while there was a concomitant increase in IL-10. Blockade of PD-L2 by antibody respectively resulted in restoration of CD4 T cell proliferation, IFN-γ production and reduced IL-10. This would suggest that PD-L2 engagement uses IL-10 to control the immune response [20].

The use of the PD1:PD-L1/L2 pathways are a common feature of the tissue dwelling helminths. PD-L1 has been shown to play a role in mediating T cell suppression during murine *Schistosoma mansoni* infection [21,22]. PD-L1 upregulation on splenic macrophages isolated from *S. mansoni* infected hosts or naïve macrophages exposed to *S. mansoni* worm *ex vivo*, induces hypo-responsiveness of naïve CD4 T-cells and CD8 T-cells. These macrophages are capable of inducing anergy in T-cells in a contact dependent manner but not IL-4-, IL-13-, IL-10, TGF-β, and NO-independent. T-cell anergy was abrogated by application of blocking antibody to PD-L1 and not PD-L2 [21]. Similarly, in murine models of cysticercosis infection, spleen cells recovered during *T. crassiceps* infection demonstrate low proliferative response to parasite-specific antigenic-stimulation suggesting down modulated T cell response [23]. Peritoneal or splenic macrophages mapped to the alternatively activated phenotype with high expression of PD-L1 and PD-L2. *in vitro* culture of these macrophages with naïve T cell suppressed T cell proliferation in contact dependent manner but not IL-10, IFN-γ and NO dependent. Moreover, blocking antibody to PD-1 restores T cell responses. While the exact use of PD-L1 or PD-L2 differs from infection to infection there is a clear pattern of the programmed death pathway to control immune responses that in some cases is dependent upon IL-10.

F. hepatica immunomodulators

Studies of the *F. hepatica* immune response and the transition to chronicity of infection has demonstrated that both host and parasite possess mechanisms to temper the response; thereby avoiding immunopathology but limiting complete parasite elimination. Secretion of immunomodulators into the host environment is a clear method of evading host immune effector mechanisms. *F. hepatica* cathepsin L1 (CL1) prevents parasite death by cleaving host immunoglobulin at the hinge region, thereby preventing antibody-dependent cell-mediated cytotoxic (ADDC) killing of fluke by host innate immune cells [24]. Additionally, CL1 suppresses mitogen-induced lymphocyte proliferation and cleaves CD4 from the surface of T cells of ovine hosts. Blocking of cathepsin activity with a cysteine protease inhibitor however, restores lymphocyte proliferation [25]. A subtler mechanism of controlling immune responses has been ascribed to peroxiredoxin (Prx). Prx promotes Th2 polarisation and activates macrophages alternatively in IL-4 and IL-13 independent pathways. Passive transfer of anti-Prx antibody or immunization of mice with recombinant Prx abrogates alternative macrophage activation and Th2 responses [26,27].

In 2011 Robinson *et al.* defined a family of small molecules, HDMs that mimic the mammalian host antimicrobial peptides (or defensins) [28•]. These interfered with LPS recognition and reduced subsequent inflammation upon LPS injection, thereby limiting innate immune responses. Further study demonstrated roles for FhHDM in altering antigen processing and presentation by preventing endosomal acidification [29]. This negative effect on endosome acidification in macrophages also impedes the IL-1β response [30]. While blocking antigen processing pathways has an obvious benefit to parasite evasion, the benefits of limiting IL-1β are less overt. None the less this demonstrates a clear case of host mimicry benefitting parasite survival. A second area in which parasite mimicry of host signalling events could be said to have occurred is within the TGF-β family.

TGF-β signalling and effects

TGF-β signalling is a pleiotropic system responsible for both control of immune responses and developmental. TGF-β is a superfamily compromising of both bone morphogenic proteins (BMP) ligands and their receptors and TGFβ ligands and their receptors. Within the immune system TGF-β can trigger fibrosis [31]; trigger Th17 differentiation [32] and mediate tolerance and regulation [33]. Developmentally, TGF-β1KO in mice gives rise to a 50% embryonic lethal phenotype due to defects in haematopoiesis and endothelial development [34], TGF-β2 and -β3 KO models also give rise to live births but death shortly afterwards due to cardiac and other abnormalities [35]. Signalling components SMAD2 [36], SAMD4 [37], and TGFRII [38] are all embryonic lethal but SMAD3 KO giving rise to live pups [39].

TGF-β amongst the parasites

Given the developmental importance of TGF-β it is not surprising to find it conserved in multiple parasites *Brugia malayi*, *Brugia pahangi* [40,41]. Indeed the *B. malayi* protein BM-TGH2 was the first of these proteins to be shown to bind the host receptor complex through the use of the MLEC luciferase assay. *Heligmosomoides polygyrus*, *Nippostrongylus braziliensis*, *Haemonchus contortus*, *Teladorsagia circumcincta* [42•], *Ancyclostoma caninum* [43], *S. mansoni*, *Schistosoma japonicum* [44•,45] and *F. hepatica* [46••].

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FhTLM

Our group initially described the *F. hepatica* TGF-like molecule (FhTLM) in 2015 [46••] after using the then unpublished genome to screen for scaffolds which possessed homology to the conserved domain of the TGF protein, initially three distinct genes were found one of which we termed FhTLM. In contrast to Hp-TGM derived from *H. polygyrus*, FhTLM appears to retain a predicted structure similar to the mammalian TGF protein. Expression analysis revealed a highly restricted pattern with NEJs expressing the highest levels of mRNA. *in situ* hybridisation analysis revealed that mRNA probes bound throughout the NEJ parasite, lacking a tissue restriction seen in the related Schistosome trematodes [44•], however this may be related to the hermaphrodite nature of *F. hepatica*. The adult had low levels of mRNA and a correspondingly restricted *in situ* expression profile. This expression data corresponded to results seen when exogenous protein was added to *ex vivo* parasite cultures. Parasite survival was improved in the NEJs but not the adults, suggesting that the components for TGF signal transduction might not be expressed within adults. Improved survival was also supported by the finding that NEJs were more active in their movement. A final developmental role was seen when eggs were incubated with exogenous protein and we observed that embryonation rate increased in the presence of FhTLM [46••].

Previous work using *H. polygyrus* has suggested that a component of parasite ES could bind to mammalian TGF-receptors as measured by a TGF-responsive reporter cell line [47••]. Moreover, Grainger *et al.* demonstrated that *H. polygyrus* ES could induce Foxp3 and suppress a Th2 allergic response within the lung. To determine if FhTLM could bind to and initiate mammalian signalling we utilised a mink lung reporter cell line (MLEC) and found that FhTLM had an effect, albeit less potent than mammalian protein [48••]. The activity of FhTLM could be inhibited by polyclonal sera which is known to cross-react with both mammalian and amphibian proteins. In line with Grainger *et al.* we found that the same anti-TGF pan species antibody could neutralise FhTLM, suggesting that while FhTLM and the molecule later described as Hp-TGM lack sequence similarity they may share some confirmation epitope [48••,49••].

We confirmed that FhTLM utilises the mammalian receptors by cloning the extracellular portions of the bovine TGFRI and TGFRII into Fc fusion proteins and demonstrating that FhTLM preferentially bound to TGFRII with greater affinity. Downstream of this we found that binding of the receptor complex causes SMAD2/3 translocation to the nucleus [48••], along with GATA1 which we also known to be important in the bovine Th2 immune response (Sulaiman *et al.* unpublished). Thereafter, we confirmed further functional relevance of FhTLM by demonstrating that like TGF-β, FhTLM also possess anti-proliferative capacity. We used both CFU forming in fibroblasts and scratch assays to demonstrate that FhTLM could delay both responses similar to TGF-β. Furthermore the use of a chemical inhibitor of the TGFRI kinase abrogated the FhTLM effect – providing evidence for specificity in the effects of FhTLM [48••].

Much work in helminth immunology has examined the impact on macrophage activation and the role of these cells during infection. What is apparent is that their roles can be multi-functional [50,51] and the route by which they are elicited diverse [52–54]. De novo generation of AAM via IL-4/IL-13 can occur in the absence of helminth antigen and indeed a different profile is obtained when helminth antigens are incorporated. In the case of *F. hepatica* an additive affect is observed in bovine AAM [55], while Prx can induce AAM-like cells in absence of IL-4/IL-13 signalling [27]. With this in mind and our knowledge of the restricted expression of FhTLM to the NEJ stage we examined the effect of FhTLM on macrophage activation. This is especially important given their potential role in protective immunity. Few mechanisms have been shown to kill *F. hepatica* NEJs of which one is antibody-dependent cell cytotoxicity [56,57] and macrophages have been shown to partake in this response [58].

Therefore, we firstly examined the effect of FhTLM on macrophage phenotype and found that FhTLM caused a slight elevation in arginase but not exceeding what we observe in IL-4 stimulated cells. IL-12 or nitric oxide were not elevated by IL-10 was increased in line with TGF-β stimulation. The most apparent change was in the expression of the mannose-receptor and PD-L1, which were both elevated above the levels induced by IL-4. Through use of siRNA against TGFRII we found that FhTLM needed an intact signalling complex to cause this response. Importantly, macrophages pre-pulsed with FhTLM lost their ability to kill NEJs in the presence of specific antibody again in a TGFRI-dependent mechanism [48••]. This anti-inflammatory effect of FhTLM is in line with recent findings showing that Hp-TGM was capable of inducing $Foxp3^{+}$ T-cells and preventing graft vs host disease [49••]. Ongoing work leads us to believe that FhTLM may have the same anti-inflammatory effect as indicated by IL-10 production (Mush-Eroje *et al. in preparation*).

Conclusions and future directions

It would appear that *F. hepatica* possesses a TGF-β mimic that can alter host responses for the benefit of parasite survival in a stage-specific manner. The ability to test this *in vivo* is dependent on the field's capacity to develop robust RNAi approaches for *F. hepatica* that would need to be conditional given the potential developmental roles of FhTLM and other known parasite TGF-like proteins. It is interesting to note that a second extremely potent anti-

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inflammatory protein, Hp-TGM, exists with comparable properties to TGF-β. Both proteins have affinity for the host receptor complex, albeit it weaker than mammalian counterparts. Interestingly for TGM this did not translate to a need for as greater concentration of parasite proteins to achieve similar effects to comparative doses of TGF-β [49••]. This might offer a clue to the origin or timing of when these anti-inflammatory effects of FhTLM arose. Our initial description of two additional unique genes fitting the profile of TGF superfamily members [46••] may also help to answer these questions. The presence of expanded gene families is a common feature of *F. hepatica* but also recently a similar phenomenon in *H. polygyrus* has been described [59•]. Should FhTLM have uses beyond parasite survival, that is as a vaccine target or xenogeneic therapeutic is an exciting but untested prospect.

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