Development of *Acanthocheilonema vitaeae* in *Meriones shawi*: Absence of microfilariae and production of active ES-62

Felicity E. Lumb | James Doonan | Marlene Corbet | Miguel A. Pineda | Margaret M. Harnett | William Harnett

1 Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, UK
2 Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, UK

Correspondence
William Harnett, Strathclyde Institute of Pharmacy and Biomedical Sciences, 161 Cathedral Street, University of Strathclyde, Glasgow G4 0RE, UK.
Email: w.harnett@strath.ac.uk

Funding Information
This work was funded by awards to WH and MMH from Arthritis Research UK (21133) and the BBSRC (BB/M029662/1 and BB/M029727/1).

Abstract
**Aims:** ES-62 is a well-studied anti-inflammatory molecule secreted by L4-adult stage *A. vitaeae*. We maintain the life cycle of *A. vitaeae* using *Meriones libycus* as the definitive host. Here, we investigated whether the full life cycle could be maintained, and functional ES-62 produced, in a related jird species—*Meriones shawi*.

**Methods and Results:** Adult worms were produced in comparable numbers in the two species, but very few microfilariae (MF) were observed in the *M. shawi* bloodstream. *M. shawi* ES-62 produced ex vivo was functional and protective in a mouse model of arthritis. Myeloid-derived cells from naïve and infected jirds of both species were compared with respect to ROS production and osteoclast generation, and some differences between the two species in both the absence and presence of infection were observed.

**Conclusions:** The life cycle of *A. vitaeae* cannot be successfully completed in *M. shawi* jirds but L3 stage worms develop to adulthood and produce functional ES-62. Preliminary investigation into jird immune responses suggests that infection can differentially modulate myeloid responses in the two species. However, species-specific reagents are required to understand the complex interplay between *A. vitaeae* and its host and to explain the lack of circulating MF in infected *M. shawi* jirds.

**KEYWORDS**
ES-62, filarial nematode, jird, microfilaria, myeloid cell, osteoclast

1 | **INTRODUCTION**

Filarial nematodes are a group of arthropod-transmitted parasites of vertebrates. Adult worms, depending on species, are found in various tissues and body cavities of their definitive hosts and produce microfilariae (MF) that circulate in the bloodstream or migrate through the tissues to enable transmission. Filarial nematodes often induce lifelong infections, due to their ability to modulate their host’s immune response to promote their own survival while at the same time, limiting host pathology. This immunoregulation is thought to be driven by the secretion of bioactive molecules by the nematodes.1 One of the best characterized secreted molecules is ES-62, a phosphorylcholine-containing glycoprotein produced by *Acanthocheilonema vitaeae*. ES-62 is secreted by the post-infective...
larva life cycle stages of the worm and can be detected in the serum of infected *Meriones libycus* jirds. ES-62 has been demonstrated to modulate the activity of multiple immune system cells such as dendritic cells, macrophages, mast cells and B and T lymphocytes [reviewed in5]. As a consequence, ES-62 has been found to protect against development of autoimmune diseases such as rheumatoid arthritis (RA;7) and systemic lupus erythematosus (SLE;8); allergic diseases such as asthma9 and, more recently, obesity-accelerated ageing,8 in mouse models.

We routinely established the full life cycle of *A. viteae* in the jird, *M. libycus*, and adult worms can be retrieved and cultured ex vivo to isolate and purify ES-62. Despite being an excellent host for *A. viteae*, our *M. libycus* colony is difficult to maintain due to the absence of a commercial source for providing new members (the colony was transferred to Strathclyde from the National Institute for Medical Research, London, in 1991) resulting in a high degree of inbreeding that makes it challenging to produce healthy offspring. *A. viteae* can also be developed in the more readily available closely related *Meriones unguiculatus*,9 but our previous use of this species resulted in production of female worms notably smaller than those developed in *M. libycus* (results not shown). We therefore explored whether we could establish the life cycle in a further closely related readily available species of jird, *Meriones shawi*, and whether nematodes from these jirds could produce functional ES-62. We were also interested in investigating the effect of ES-62 on the jird immune response. Despite having extensively studied the effect of ES-62 in mouse models of disease, we know very little about how the molecule interacts with the immune system on the jird immune response. Work in this area has been hampered by the absence of jird-specific reagents required to investigate the effect of ES-62 in mouse models of disease, we know very little about how the molecule interacts with the immune system of a receptive host. Work in this area has been hampered by the lack of jird-specific reagents required to investigate the effect of ES-62 on specific immune cell populations; however, we were able to study the effects of the nematode product on certain functional responses of host cells.

2 METHODS

### 2.1 Acanthocheilonema viteae life cycle

The *Acanthocheilonema viteae* life cycle was maintained in *Meriones libycus* and in soft ticks (*Ornithodoros moubata*), as described previously.10 Briefly, adult male jirds were infected subcutaneously with 120 arthropod-derived larvae (L3) which matured into adult worms over the course of 3 months. The number of MF was then determined in a small blood sample (5 µL), and jirds with an appropriate level of MF (approximately 120 MF in 5 µL blood) were used to infect ticks to complete the life cycle. Adult worms were obtained from jirds by dissection from under the skin of jird pelts following exsanguination using CO₂ and counted and sexed—female worms were identified as larger nematodes while males are shorter and have a ‘corkscrewed’ phenotype. The worms were cultured ex vivo in ‘complete’ RPMI 1640 medium (containing 50 U/mL penicillin and 50 µg/mL streptomycin, supplemented with 10% glucose [Life Technologies]) for generation of ES-62 as described previously.5–8 The same procedures were applied to *Meriones shawi*.

### 2.2 In vitro macrophage differentiation

Bone marrow (BM) cells were collected from uninfected (‘naïve’) or infected *M. libycus* or *M. shawi* and cultured in ‘complete’ DMEM medium containing 50 U/mL penicillin, 50 µg/mL streptomycin and 10% FCS, supplemented with 20% cell supernatant from the M-CSF producing cell line L929. Fresh medium containing 20% L929 cell supernatant was added on day 4, and derived macrophages were used in experiments on day 7. BM-derived macrophages (BMMs) were scraped in ‘complete’ RPMI 1640 medium, plated into 96-well tissue culture plates, left to adhere overnight and then stimulated with *Salmonella enterica* LPS (0.1 µg/mL) or CpG-ODN1826 (5 µmol/L) for 24 hours for functional assays.

### 2.3 Reactive oxygen species (ROS) flow cytometry assay

The presence of ROS in BM monocytes and BMMs was measured using 2,7'-dichlorofluorescein diacetate (DCF-DA, Sigma). Briefly, ROS in BM monocytes was measured following red cell lysis, while ROS in BMMs was measured after stimulation with pathogen-associated molecular pattern (PAMP) molecules for 24 hours. Cells were incubated with 50 µmol/L DCF-DA for 30 minutes at 37°C and then washed in Fluorescence-activated cell sorter (FACS) buffer (2.5% BSA; 0.5mM EDTA, in PBS) to stop the reaction. Data were acquired using a FACS Canto flow cytometer and analysed using FlowJo Software (Tree Star Inc, OR, USA, version 8.8.7). Populations were gated using non-DCF-DA stained controls.

### 2.4 In vitro osteoclast differentiation

Osteoclasts (OCs) were differentiated from naïve or infected *M. libycus* or *M. shawi* BM as previously described using mouse M-CSF and RANKL reagents.10 BM was assessed for OC differentiation by TRAP staining (Leukocyte Acid Phosphatase Kit, Sigma) on day 6, and cells that stained positive for TRAP with ≥3 nuclei were counted as OCs. Images were obtained on an EVOS FL Auto Cell Imaging System at 20x magnification with scale bars set at 200 µm.

### 2.5 Collagen-induced arthritis model

Male DBA/1 mice (8-10 weeks; Harlan Olac), for the collagen-induced arthritis (CIA) model,5,11 were maintained at the Central Research Facility at the University of Glasgow (UoG) in accordance with the Ethics Review Board of the UoG. CIA was induced by intradermal
immunization of mice with bovine Collagen Type II (CII) emulsified with complete Freud’s adjuvant (MD Biosciences) on day 0, and animals challenged with CII in PBS (intraperitoneal injection) on day 21. Mice were treated subcutaneously with PBS or M shawi purified, endotoxin-free ES-62 (2 µg/dose) on days −2, 0 and 21. Joint damage (articular score) was scored as previously described.5,11 Draining lymph nodes from CIA and ES-62-treated CIA mice were analysed, and lymphocytes identified based on size and granularity (n = 3; F). Each data point represents an individual animal and is presented as mean ± SEM. Two-tailed Student’s t tests were used to analyse species differences, where * = P < .05 and *** = P < .001.

FIGURE 1 The Acanthocheilonema viteae life cycle cannot be completed in Meriones shawi. Age-matched male M libycus and M shawi jirds were infected with 120 L3 A. viteae larvae by sub-cutaneous injection. Adult worms were dissected from jird pelts following exsanguination using CO2 and counted and sexed (A M libycus—n = 36; M shawi—n = 35). The presence of microfilariae (MF) was determined by blood sampling 5 µL and counting MF in each sample (B). Linear regression modelling was used to compare MF counts to adult worm burden, and the association of worm burden and MF count was confirmed in M libycus (P = .02) but not M shawi jirds (C). Infected M libycus (n = 31) and M shawi (n = 18) jirds were weighed (grams) after 3 mo of infection (D). ES-62 purified from adult worms from M shawi jirds was tested for therapeutic properties in the mouse CIA model of arthritis with disease shown as mean arthritic score (ES-62 n = 5; PBS n = 6; E). Draining lymph nodes from CIA and ES-62-treated CIA mice were analysed, and lymphocytes identified based on size and granularity (n = 3; F). Each data point represents an individual animal and is presented as mean ± SEM. Two-tailed Student’s t tests were used to analyse species differences, where * = P < .05 and *** = P < .001.

LSD post-test, two-way ANOVA with Tukey’s multiple comparisons and linear regressions where appropriate. In all cases, *P < .05, **P < .01 and ***P < .001.

3 | RESULTS

3.1 | Acanthocheilonema viteae fecundity is host sub-species specific

Acanthocheilonema viteae is considered to only fully mature in a limited range of hosts of the rodent family Gerbillae, for example M libycus and Gerbillus hirtipes.12 We investigated whether a closely related species M shawi would be suitable as a definitive host for these parasites. Adult male M libycus and M shawi were infected with 120 L3 larvae derived from the tick intermediate host. Adult male and female worms were successfully obtained from both species with

2.6 | Statistics

All data were analysed using GraphPad Prism 6 software, employing statistical analysis by Student’s t test, one-way ANOVA with Fisher’s LSD post-test, two-way ANOVA with Tukey’s multiple comparisons and linear regressions where appropriate. In all cases, *P < .05, **P < .01 and ***P < .001.
no significant difference in numbers (Figure 1A) or length of worms. However, the number of MF found in the blood of infected *M. shawi* was significantly lower than the level in *M. libycus* (Figure 1B), to the point of being essentially zero. Unlike some other filarial nematode species, we are not aware of periodicity having been described for MF of *A. viteae*, but in any case examination of blood samples from infected *M. shawi* at three different time points (~06.00, 11.00-12.00 [our usual time] and 20.00-22.00) over a number of days also did not lead to MF being detected. Moreover, examination of the peritoneal and pleural cavities of infected *M. shawi* likewise failed to detect MF. Interestingly, in *M. libycus* jirds the number of worms was significantly correlated with the number of MF, but this was not recapitated in *M. shawi* jirds (Figure 1C). Indeed, it was not possible to infect ticks from the latter species (results not shown) and so the full *A. viteae* life cycle cannot be completed in *M. shawi* jirds. Although these species are closely related, their significant difference in weight suggests that there may be a number of outward physical and perhaps physiological differences between these species (Figure 1D) but whether this has any impact on circulating MF numbers is unknown.

Similarly to adult worms from *M. libycus*, worms from *M. shawi* could be cultured in vitro for several weeks and comparable levels of ES-62 purified from culture medium. The ES-62 isolated from worms derived from *M. shawi* and *M. libycus* was compared on both denatured (SDS) and native PAGE gels and found to demonstrate the same molecular weights from the two species (data not shown). In order to determine whether the ES-62 purified from adult worms from *M. shawi* had similar immunomodulatory properties to ES-62 obtained from *M. libycus*, we tested the former in the CIA model of rheumatoid arthritis. As observed previously for *M. libycus*-derived ES-62, mice treated with *M. shawi* ES-62 were significantly protected against development of pathology in this model (Figure 1E). Additionally, treatment with *M. shawi* ES-62 was able to significantly suppress lymphocyte activation following ex vivo stimulation compared to control mice, confirming that *M. shawi* ES-62 was immunomodulatory in the model (Figure 1F).

### 3.2 | *Acanthocheilonema viteae* trains host myeloid cell responses in *Meriones libycus*

*Meriones shawi* adult worms were found to produce live MF when cultured ex vivo, and examination of these by light microscopy revealed no obvious difference from those produced by females developed in *M. libycus*. Also, although we did not quantitate MF release, examination of culture flasks indicated that the *M. shawi* cultures contained MF levels which appeared to be no different to the (usual) high numbers present in the *M. libycus* cultures. We therefore wished to investigate whether the low level of MF observed in the blood of *M. shawi* was due to increased immune responses against the parasite. Due to a lack of species-specific reagents, we have examined ubiquitous markers of inflammation as a preliminary attempt to measure aspects of the immune response to the nematode in *M. shawi* and *M. libycus*. First, we evaluated the level of intracellular ROS in BM cells from both species of jird using flow cytometry. We identified a monocyte-like population of large cells with an intermediate granularity that produced ROS (Figure 2A) and found a significantly higher percentage of these cells in BM from *M. shawi* compared to *M. libycus* (Figure 2B). When we separated these cells based on expression of intracellular ROS, we observed that monocytes from *M. shawi* have increased percentage of ROS(high) cells compared to *M. libycus* although this only reached statistical significance between naïve, but not infected, animals (Figure 2C). Infection did not impact on the percentage of these cell populations in either jird species (Figure 2B and C). Thus, to try and further address the effect of *A. viteae* on host immune responses we differentiated BM cells from naïve and infected *M. shawi* and *M. libycus* jirds into macrophages (BMMs) and measured their ROS production after stimulation with PAMPs. Previously we have shown that in vivo treatment with ES-62 can modulate in vitro host (mouse) BMMs by reducing pro-inflammatory cytokine expression in response to PAMPs.

### 4 | DISCUSSION

*M. shawi* was found to be an appropriate host for production of adult *A. viteae* and subsequently, for generation of ES-62 that was found to be biologically active. Despite the presence of viable adults, infected *M. shawi* jirds had very low levels of circulating MF in their bloodstream, meaning the full nematode life cycle could not be completed in this species. There are a number of potential explanations for the lack of MF despite the development of viable adult worms: that the MF are being targeted by the immune response of the *M. shawi* jirds, that the female adults are unable to produce MF, that the MF are located in a different anatomical site or that MF only periodically enter the bloodstream. On checking, we could find no
clear evidence for the last two options and as living MF were observed in the medium when adult worms from *M. shawi* were cultured in vitro and that were not apparently different either quantitatively or qualitatively from MF released by females derived from *M. libycus* when examined by light microscopy, there is clearly not a problem with fecundity.

With respect to immune responses, we did not attempt to measure antibodies against MF due to lack of reagents; however, previous studies in the model mouse host have shown that *A. viteae* MF may be targeted by them. Nonetheless, interestingly, we found that *M. shawi* jirds have a greater percentage of ROS high monocytes and *M. libycus* jirds have a greater percentage of ROS high monocytes than *M. libycus* jirds suggesting that some immune responses may differ between the two species. However, infection had no effect on the percentage of such cells in either species. Nonetheless, infection with *A. viteae* significantly reduced the production of ROS in response to bacterial PAMPs in BMM from *M. libycus*, but not *M. shawi*, jirds. Although the latter produce much lower levels of ROS when stimulated, possibly making differences more difficult to detect, this is a finding that may be worthy of further investigation relating to whether it impacts on MF numbers. Alternatively, it could be that the differences in myeloid responses observed in infected *M. libycus* and *M. shawi* may be influenced by the lack of circulating MF in *M. shawi* rather than the infection per se. Recovery of cellular responsiveness in filariasis patients treated with microfilaricidal drugs such as DEC support the idea that MF play a key role in the modulation of the host immune response to the parasite. Indeed, stimulation in vitro with *Brugia malayi* MF lysate induced a regulatory phenotype in monocytes and macrophages characterized by expression of IL-10 and PD-L1. It is possible that the MF are reducing the potential for ROS generation in immune cells derived from *M. libycus* as a mechanism to ensure their own survival. MF may be able to travel throughout the body including the BM and so could be having local effects in this ‘organ’, perhaps in some way accounting for the differences we see in osteoclast numbers differentiated from BM between jird species.

Any biological significance of the striking differential effect of infection on numbers of osteoclasts in the two jird species remains to be established and may be influenced by the relative ability of the two species—an observation that remains to be explored and explained—to differentiate osteoclasts in the absence of infection. These cells are primarily involved in maintenance of healthy bones but can adopt a pathogenic phenotype in diseases like rheumatoid
arthritis. As mentioned earlier, \textsuperscript{11} ES-62 can inhibit osteoclastogenesis in protecting against joint disease in the CIA model of RA, but the differential effect observed in the two jird species suggests it may not be active in this way in the present study, at least in \textit{M shawi}.

CONFLICT OF INTEREST
The authors have no conflicts of interest.

AUTHOR CONTRIBUTIONS
JD and FL contributed equally to this manuscript, maintained the \textit{Acanthocheilonema viteae} life cycle, and performed the experiments for the study that JD, FL and WH conceived. MC, MAP and MMH conceived, performed and analysed the mouse arthritis model. All authors were involved in reviewing and revising the manuscript and have approved the final version.

DISCLOSURES
None.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID
William Harnett https://orcid.org/0000-0001-9545-9401

REFERENCES
1. Harnett MM, Harnett W. Can parasitic worms cure the modern world’s ills? Trends Parasitol. 2017;33:694-705.
2. Harnett W, Worms MJ, Kapil A, Grainger M, Parkhouse RM. Origin, kinetics of circulation and fate in vivo of the major excretory-secretory product of \textit{Acanthocheilonema viteae}. Parasitology. 1989;99:229-239.
3. Stepek G, Auchie M, Tate R, et al. Expression of the filarial nematode phosphorylcholine-containing glycoprotein, ES-62, is stage specific. Parasitology. 2002;125:155-164.
4. Pineda MA, Lumb F, Harnett MM, Harnett W. ES-62, a therapeutic anti-inflammatory agent evolved by the filarial nematode \textit{Acanthocheilonema viteae}. Mol Biochem Parasitol. 2014;194:1-8.
5. McLnnes IB, Leung BP, Harnett M, Gracie JA, Liew FY, Harnett W. A novel therapeutic approach targeting articular inflammation using the filarial nematode-derived phosphorylcholine-containing glycoprotein ES-62. J Immunol. 2003;171:2127-2133.
6. Rodgers DT, McGrath MA, Pineda MA, et al. The parasitic worm product ES-62 targets myeloid differentiation factor 88-dependent effector mechanisms to suppress antinuclear antibody production and proteinuria in MRL/lpr mice. Arthritis Rheumatol. 2015;67:1023-1035.
7. Rzepecka J, Siebeke I, Coltherd JC, et al. The helminth product, ES-62, protects against airway inflammation by resetting the Th cell phenotype. Int J Parasitol. 2013;43:211-223.
8. Crowe J, Lumb FE, Doonan J, et al. The parasitic worm product ES-62 promotes health- and life-span in a high calorie diet-accelerated mouse model of ageing. PLoS Pathog. 2020;16(3):e1008391.
9. Mossinger J, Barthold F. Fecundity and localisation of Dipetalonema viteae (Nematoda, Filarioidea) in the jird Meriones unguiculatus. Parasitol Res. 1987;74:84-87.
10. Worms MJ, Terry RJ, Terry A. Dipetalonema witei, filarial parasite of the jird, \textit{Meriones libycus}. I. Maintenance in the Laboratory. J Parasitol. 1961;47:963-970.
11. Doonan J, Lumb FE, Pineda MA, et al. Protection against arthritis by the parasitic worm product ES-62 and its drug-like small molecule analogues is associated with inhibition of osteoclastogenesis. Frontiers Immunol. 2018;9:1016.
12. Schrempf-Eppstein B, Kern A, Textor G, Lucius R. \textit{Acanthocheilonema viteae}: vaccination with irradiated L3 induces resistance in three species of rodents (\textit{Meriones unguiculatus, Mastomys coucha, Mesocricetus auratus}). Trop Med Int Health. 1997;2:104-112.
13. Hawking F. The 24-hour periodicity of microfilariae: biological mechanisms responsible for its production and control. Proc R. Soc Lond B. 1967;169:59-76. Parasitol Res 1987; 74: 84-87.
14. Goodridge HS, McGuiness S, Houston KM, et al. Phosphorylcholine mimics the effects of ES-62 on macrophages and dendritic cells. Parasit Immunol. 2017;29:27-137.
15. Gatrill AJ, Kee J, Behnke J, Wakelin D. \textit{Acanthocheilonema viteae} in mice: differences in the relative binding of microfilarial surface-specific antibody may explain the contrasting response phenotypes of BALB/c and C57/Bl10. J Helminthol. 1991;65:211-218.
16. O’Regan NL, Steinfeldt S, Venugopal G, et al. Brugia malayi microfilariae induce a regulatory monocyte/macrophage phenotype that suppresses innate and adaptive immune responses. PLoS Negl Trop Dis. 2014;8(10):e3206.
17. Pradhan S, Lahiri VL, Elhence BR, Singh KN. Microfilaria of \textit{Wuchereria bancrofti} in bone marrow smear. Am J Trop Med Hyg. 1976;25:199-200.

How to cite this article: Lumb FE, Doonan J, Corbet M, Pineda MA, Harnett M, Harnett W. Development of \textit{Acanthocheilonema viteae} in \textit{Meriones shawi}: Absence of microfilariae and production of active ES-62. Parasite Immunol. 2020;00:e12803. https://doi.org/10.1111/pim.12803