BRIEF REPORT

REVISED Current status of immunodeficient mouse models as substitutes to reduce cat and dog use in heartworm preclinical research [version 2; peer review: 4 approved]

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v2 **First published:** 17 May 2024, **13**:484
https://doi.org/10.12688/f1000research.149854.1
Latest published: 11 Sep 2024, **13**:484
https://doi.org/10.12688/f1000research.149854.2

Abstract

Chemoprophylactic prevention of veterinary heartworm disease in companion animals, caused by the vector-borne nematode parasite *Dirofilaria immitis,* is a multi-billion-dollar global market. Experimental use of cats and dogs in preclinical heartworm drug testing is increasing due to evolving drug-resistance to frontline macrocyclic lactones and renewed investment in alternative preventative drug research. We and others recently published data demonstrating proof-of-concept of utilising lymphopenic severe-combined immunodeficient (SCID) or Recombination Activating Gene (RAG)2 deficient mice with additional knockout of the IL-2/7 receptor gamma chain (γc) as alternative preventative drug screening research models of dirofilariasis. Here we summarise the current knowledge of candidate immunodeficient mouse models tested, including a comparison of susceptibility using different background strains of mice, different *D. immitis* isolates, following use of anti-inflammatory treatments to further suppress residual innate immunity, and efficacies achieved against different reference anthelmintics. We supplement this precis with new data on treatment response to the veterinary anthelmintic, oxfendazole, and initial evaluation of *D. immitis* susceptibility in CB.17 SCID and C57BL/6 RAG2$^{-/-}$γc$^{-/-}$ mice. We conclude that in addition to NSG and NXG mice, RAG2$^{-/-}$γc$^{-/-}$ mice on either a BALB/c or C57BL/6 background offer an alternative screening model option, widening access to academic and commercial laboratories wishing to pursue initial rapid *in vivo* drug screening

Open Peer Review

Approval Status ✓ ✓ ✓ ✓

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version 2 (revision) 11 Sep 2024				
version 1 17 May 2024	✓ view	✓ view	✓ view	✓ view

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whilst avoiding potentially unnecessary cat or dog testing.

Keywords
Dirofilariasis, heartworm, parasitology, anthelmintic, anti-parasitic drugs

This article is included in the NC3Rs gateway.

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Competing interests: CF, UD, AM, SM, and JM are employed by TRS Laboratories Inc. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Grant information: This research was funded by The UK National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3R) Project Grant to JDT and MJT (NC/S001131/1), NC3R Skills & Knowledge Transfer Grant to JDT (NC/W000970/1), and a Wellcome Trust Institutional Translational Partnership Award (2195760/Z/19/Z, internal award reference - iTPA2212) to JDT.
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

How to cite this article: Dagley JL, DiCosty U, Fricks C *et al.* **Current status of immunodeficient mouse models as substitutes to reduce cat and dog use in heartworm preclinical research [version 2; peer review: 4 approved]** F1000Research 2024, **13**:484 https://doi.org/10.12688/f1000research.149854.2

First published: 17 May 2024, **13**:484 https://doi.org/10.12688/f1000research.149854.1

REVISED **Amendments from Version 1**

The manuscript has been amended with additional information based on reviewers' critique and to incorporate new data from an additional publication arising after original submission. Edits to the original manuscript include the addition of recent data by Risch *et al.* (2024), having demonstrated Missouri isolate *Dirofilaria immitis* adult development in B.6 RAG2$^{-/-}$/yc$^{-/-}$ mice (Table 1). Additionally, we have discussed the development of caval syndrome in B.6 RAG2$^{-/-}$/yc$^{-/-}$ mice, the presence of pending or awarded patents in the UK and other territories, and touched on the indication of an ancillary requirement for the host adaptive immune responses to deliver optimum macrocyclic lactone efficacy.

Any further responses from the reviewers can be found at the end of the article

Research highlights

Scientific benefits(s):

- A variety of immunodeficient mouse models of *Dirofilaria immitis* (heartworm) are reproducibly susceptible to tissue-phase L4 stage larvae.
- Oxfendazole's effectiveness in reducing *D. immitis* tissue-phase larvae demonstrates potential use as a heartworm preventative.

3Rs benefits(s):

- As alternative *in vivo* models for heartworm, mice have the potential to reduce the overall use of specially protected species, cats and dogs, in heartworm preventative compound screening.
- Mice present with no clinical signs of tissue-phase *D. immitis* infection over 5 weeks, categorising this model as a 'mild procedure'.
- Mouse models have the potential to be used as a screening model before moving onto more sentient and highly protected species, potentially reducing the number of chronic procedures by 67% and longitudinal infection studies risking moderate to severe welfare arising in cats and dogs.

Practical benefits(s):

- The use of rodent heartworm models has advantages in comparison to cats and dogs for preliminary drug screening such as ease of pharmacology standardisation, reduced costs of maintenance and higher throughput.
- Increased variety of commercially available mouse strains susceptible to heartworm extends global access for heartworm drug testing in laboratories where heartworm infectious larvae can be supplied.

Current applications:

- Evaluation of anti-*Wolbachia* compounds at our laboratory, as a novel approach to heartworm prevention.
- Adoption in industry labs for more widespread use as an initial *in vivo* screening model for preventative research and development.

Potential applications:

- Onward use of models in other basic and applied biological research e.g. heartworm developmental biology, mechanisms of drug resistance, drug repurposing, immune-mediated control of heartworm including vaccine research and biomarker discovery.

Introduction

Affecting felids and canids, heartworm disease is caused by the mosquito-borne filarial nematode, *Dirofilaria immitis.* With vectors including the invasive *Aedes albopictus,* heartworm has an emerging global distribution (Simón *et al.*, 2012; Noack *et al.*, 2021; Morchón *et al.*, 2022). Canine chronic-progressive heartworm disease can result in heart failure following establishment of adult worms within the pulmonary vascular system. In cats, immature worms can result in potentially lethal heartworm-associated respiratory disease (McCall *et al.*, 2008). Humans are at risk of developing abbreviated zoonotic infections, with increasing reported incidence (Reddy, 2013). Human pulmonary lesions formed by infections are frequently confused with tumours (Saha *et al.*, 2022). A related subcutaneous parasite, *D. repens,* is also widespread in Europe and Asia, risking renal damage in dogs and zoonotic ocular-dermal pathologies (Genchi and Kramer, 2017; Noack *et al.*, 2021).

Drugs available for safe prevention and post-diagnosis treatment of heartworm disease are limited. The arsenical injectable, melarsomine, is the only registered cure for adult heartworm (Self *et al.*, 2019; Morchón *et al.*, 2022). Melarsomine is not registered for use in cats and risks severe adverse events in dogs, requiring complex protracted case management, exercise restriction and supplementary treatments. Comparatively, primary control of heartworm relies on chemoprophylaxis using macrocyclic lactones (ML). Despite high efficacy of MLs during the first 60 days of *D. immitis* infection, concerns have been raised regarding the development of resistant isolates following their widespread utilisation within veterinary medicine. Resistance of *D. immitis* has been formally demonstrated within both field and laboratory settings, with "JYD-34" and "ZoeLA" isolates identified as ivermectin-resistant by laboratory-based validation (McTier *et al.*, 2019; Prichard and Geary, 2019). Thus, there is a growing need for new heartworm chemoprophylactic drugs utilising a novel mode of action (Turner *et al.*, 2020).

Until recently, only laboratory-reared cats and dogs have been validated for *in vivo* drug screening of preventative heartworm drug compounds following experimental infections. Ethical concerns arise following the use of such highly sentient animals, categorised with non-human primates as specially protected species under UK law. Additionally, to satisfy regulatory requirements that new prophylactic formulations prevent arrival of adult worms in the heart and lungs, studies are necessarily lengthy (≥6 months) and are vulnerable to moderate to severe complications. This is particularly evident in experimental cat infections due to potentially lethal immune-pathological respiratory disease when immature worms die in the lungs (Dillon *et al.*, 2017). Finally, using cats and dogs faces practical challenges of keeping large laboratory-bred animals for long time-periods and limits throughput for drug development. Our analysis of published experimental heartworm studies between 2015-2020 identified that 1324 lab-reared cats and dogs have been documented in heartworm experimental research (221 per annum) with the majority (63%) used in drug testing.

Following previous success by our laboratory and others in developing rodent models for the medically important filarial nematodes *Brugia malayi, Onchocerca volvulus,* and *Loa loa* (Halliday *et al.*, 2014; Pionnier *et al.*, 2019; Marriott *et al.*, 2022), investigation into the permissiveness of mice to *D. immitis* has recently been demonstrated by our laboratories (Marriott *et al.*, 2023) and independently by Hess *et al.* (2023).

Here we summarise the status of heartworm immunodeficient mouse models in terms of *D. immitis* isolates, strains of inbred mutant and genetically modified mice, infection durations, and validations of drug testing models in terms of different anthelmintic efficacies. We supplement prior data with new findings demonstrating Georgia III strain *D. immitis* recoveries from NSG mice do not significantly vary at 5 weeks in comparison to earlier time points, and further validate a five-week drug screen, with single daily drug exposures at 4-week intervals using a novel reference veterinary filaricide, oxfendazole. We report evaluations of two additional commercially available mouse strains, C57BL/6NTac.Cg-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Wjl} (RAG2$^{-/-}$γc$^{-/-}$), and C.B-*Igh-1*^{b}/IcrTac-*Prkdc*^{scid} (C.B-17 SCID) with or without additional steroid treatment, for susceptibility to *D. immitis* tissue-phase larval infection.

Methods

Animals

Male NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were purchased from Jax Labs, USA. Male C57BL/6NTac.Cg-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Wjl} (B.6 RAG2$^{-/-}$/γc$^{-/-}$) and C.B-*Igh-1*^{b}/IcrTac-*Prkdc*^{scid} (C.B-17 SCID) mice were purchased from Taconic, USA. Mice were 5-7 weeks old and 20-30g at the start of study. All mice were group housed at TRS Labs and allowed minimum seven days acclimation before study, kept in stacked cages and with access to food and water *ad libitum.*

Dirofilaria immitis L3 production

Dirofilaria immitis Georgia III (GAIII) isolate microfilariae in dog blood were fed to *Aedes aegypti* female mosquitoes (Liverpool strain) using a glass feeder at a density of 1,000-2,500 mf/ml with third-stage infective larvae (L3) collected 14 days later following protocols by Marriott *et al.* (2023). Mosquitoes were kept at temperatures 75-80°F, humidity at 72-95%.

Animal infections and dosing

NSG mice (Figure 1A) were subcutaneously inoculated with 100 GAIII *Di*L3 into the flank and maintained until 5 weeks post-infection. Animals were allocated to treatment groups via cage (non-randomised), due to logistical constraints, experimental unit being a single animal. Treatment group (n=4 mice) received oral 5mg/kg bi-daily dose of oxfendazole (d1+d29). Oxfendazole was suspended in standard suspension vehicle (SSV; 0.5% carboxymethyl cellulose, 0.5% benzyl alcohol, 0.4% tween 80, 0.9% NaCl). B.6 RAG2$^{-/-}$/γc$^{-/-}$ (n=5 mice) and C.B-17 SCID (n=5 mice) (Figure 2A) received a subcutaneous injection of 2mg methylprednisolone acetate (MPA) in 200uL ddH_2O. Control groups received a matching volume of ddH_2O. Dosing was immediately followed by subcutaneous inoculation of 200 GAIII *Di*L3 into the

flank. MPA dosing was repeated on d7, and mice sustained until 14 days post-infection (no blinding used during inoculation or dosing). All mice were monitored daily for welfare and weighed weekly, no animals excluded during the study. Those monitoring and weighing animals were aware of group allocation.

Parasite collection

Mice were humanely euthanised by schedule one (rising CO_2) two or five weeks post-infection, dependent on study design (Figure 1A; Figure 2A). Collection and visual quantification of fourth-stage larvae (L4) using a light microscope followed protocols by Marriott *et al.* (2023).

Statistical analysis

For deriving group size for drug testing, we utilised data of average yield and variation of GAIII larvae at 4 weeks post-infection in NSG mice (27.3±6.2, n=5) (Marriott *et al.*, 2023) to calculate effect size and statistical power of minimum 70% reduction by drug treatment (e.g. predicted mean number of L4 larvae=8.2±1.9, d=4.2, power>0.9, n=3 per group, 2-tailed independent T test, alpha=0.05, calculated in G*Power 3.1). We included an additional mouse per group as mitigation in case of early welfare issues.

Tests were performed using GraphPad Prism 9.1.2. D'Agostino and Pearson omnibus Shapiro-Wilk normality testing indicated non-parametric analyses. Mann-Whitney tests or Kruskal Wallis with Dunn's *post hoc* tests were used to compare quantitative differences. Chi-square tests for trend were used to assess categorical data over time. Statistical significance was defined as $P \leq 0.05$, experimental unit being a single animal. Group allocation was not specified to those conducting data analysis.

Results

GAIII *D. immitis* persist for five weeks and are susceptible to oxfendazole in NSG mice

Our laboratories previously demonstrated viable L4 larval yields two-to-four weeks post-infection using NSG or NXG mouse strains, following infection with Missouri (MO) or GAIII *D. immitis* (Table 1) (Marriott *et al.*, 2023). Hess *et al.* (2023) also demonstrated permissiveness of NSG mice to the MO isolate and the ML-resistant isolate, JYD-34, extending evaluations up to six weeks (summarised in Table 1). We therefore investigated the ability of NSG mice to sustain GAIII isolate infections for five weeks (Figure 1A). Following subcutaneous inoculations of 100 *Di*L3, we reproducibly recovered GAIII *D. immitis* L4 on d35 dpi (4/4), median recovery rate of 14.5% (range 6-26%). This was not significantly variable when compared with GAIII L4 yields priorly attained at 2-4 weeks post-infection by Marriott *et al.* (2023) (Figure 1B). At five-weeks, the majority (61%) of larvae were recovered from muscle tissues (Figure 1C). Comparing to prior data at 14, 21 and 28-days post-infection, a significant linear increase in GAIII *D. immitis* developing larvae migration into muscle tissues over time was apparent (chi-square test for trend, 30.3, P<0.0001, Figure 1C). With the advantage that the impact of two daily chemoprophylactic exposures spaced 4 weeks apart could be evaluated within this extended timeframe in future studies (emulating monthly oral exposures in cats and dogs), we tested efficacy of a 5 mg/kg bid oral regimen of the benzimidazole oxfendazole, selected based on recent evidence it can mediate curative efficacies after short-course exposures in a filariasis infection model (Hubner *et al.*, 2020). After two exposure cycles (d1+d29), oxfendazole mediated a median 90% reduction in *D. immitis* L4 compared with controls (d35), curing two out of four mice (Figure 1D). Efficacy was comparable to reductions in larvae in MO, GAIII and JYD isolates treated with macrocyclic lactone regimens in NSG mice (data summarised in Table 2). Over the 35-day infection course, mice displayed no adverse behavioural changes determined during daily anecdotal observations by a veterinarian, and gained weight (Figure 1E), indicating infections and drug dosing did not cause overt clinical welfare signs.

B.6 RAG2$^{-/-}$/γc$^{-/-}$ and CB.17 SCID mice are susceptible to GAIII *D. immitis*

Hess *et al.* (2023) described mouse susceptibility to MO isolate *D. immitis* as specific to the NSG line, as other strains including lymphopenic SCID mice on NOD or B.6 backgrounds were refractory to infection (summarised in Table 1). NOD mice have inherent strain-specific deficiencies in the complement system (Verma *et al.*, 2017) and thus combinations of these, or other background strain-specific immune gene mutations, combined with susceptibility of the introduced SCID mutation and IL-2Rγ ablation, may culminate in multiple immune-impairments sufficient to allow *D. immitis* survival and growth. However, in our prior study (Marriott *et al.*, 2023), we identified compound lymphopenic (RAG2$^{-/-}$) and IL-2Rγ deficiencies on a BALB/c background as susceptible to the *D. immitis* MO isolate at two weeks, with methyl-prednisolone (MPA) steroid treatment augmenting larval recoveries. We therefore examined two commercially accessible, alternative lymphopenic mouse strains on distinct genetic backgrounds: B.6 RAG2$^{-/-}$/γc$^{-/-}$ and CB.17 SCID (BALB/c congenic), evaluating *D. immitis* GAIII L4 larval recoveries at 14dpi in groups of five mice with or without MPA treatment (Figure 2A). Whilst all (5/5) B.6 RAG2$^{-/-}$/γc$^{-/-}$ mice had recoverable *D. immitis* L4 larvae two weeks post-infection, only 2/5 CB.17 SCID mice were infection positive (Figure 2B). Yields were significantly higher in RAG2$^{-/-}$/γc$^{-/-}$ mice (median=8%, range=4-35% vs median=0%, range 0-4%, Kruskal Wallis One-Way ANOVA

Table 1. Summary of *D. immitis* isolates and mouse strains tested as suitable tissue-phase canine heartworm larval infection models.

Model background strain (Supplier)	Maximum time post-infection evaluated yield (Median % inoculate recovered) (Range, n)*				
	MO *Di*L3 (LSTM UK)	MO *Di*L3 (UKB DE)	GAIII *Di*L3 (TRS USA)	MO *Di*L3 (TJU USA)	JYD-34 *Di*L3 (TJU USA)
Wild-type (C57BL/6J)[1] B.6 (Charles River, Jackson Labs)				42dpi 0% (0-0, n=5)	
Wild-type (NOD/ShiLt)[1] NOD (Jackson Labs)				42dpi 0% (0-0, n=5)	
SCID (NOD.Cg-Prkdcscid)[1] NOD (Jackson Labs)				42dpi 0% (0-0, n=5)	
NSG (NOD.Cg- *Prkdc* ^{scid} *Il2rg* ^{tm1Wjl}/SzJ)[1,2,3] NOD (Charles River, Jackson Labs)	14dpi 5% (2-24, n=21)		35dpi 15% (6-28, n=5)	42dpi[3] 30% (1-25, n=35)	42dpi[3] 28% (8-30), n=21)
NXG (NOD- *Prkdc* ^{scid}-*IL2rg* ^{Tm1}/Rj)[2] NOD (Janvier)	14dpi 6% (1-23, n=18)				
SCID (C.B- *Igh-1* ^{b}/IcrTac- *Prkdc* ^{scid})[4] CB.17 (Charles River, Taconic)			14dpi 1.5% (0-7, n=5)		
SCID (+MPA)[4] CB.17 (Charles River, Taconic)			14dpi 0% (0-4, n=5)		
SCID (B6.Cg-PrKdcscid/SzJ)[1] B.6 (Jackson Labs)				42dpi 0% (0-0, n=5)	
RAG2γc (C;129S4- *Rag2* $^{tm1.1Flv}$*Il2rg* $^{tm1.1Flv}$/J)[2] BALB/c (Charles River, Jackson Labs)	14dpi 1.5% (0-3, n=5)				
RAG2γc (+MPA)[2] BALB/c (Charles River, Jackson Labs)	14dpi 6% (4-14, n=5)				
RAG2γc (C57BL/6NTac.Cg- *Rag2* ^{tm1Fwa} *IL2rg* ^{tm1Wji}/Rj)[4,5] B.6 (Taconic, Janvier)		180dpi 2% (0-8, n=4)	14dpi 8% (4-35, n=5)		
RAG2γc (+MPA)[4] B.6 (Taconic, Janvier)			14dpi 5% (2-22, n=5)		

[1]Data from Hess *et al.* (2023). Data quantified from graphical representations.
[2]Data from Marriott *et al.* (2023).
[3]Susceptibility reported by Hess *et al.* (2023) up to 15 weeks, but no quantitative data is available after 42dpi.
[4]TRS USA previously unpublished data.
[5]Data from Risch *et al.* (2024). Data quantified from graphical representations. Data shown as '180dpi', however nematodes may have been collected from 161-180dpi.
*Maximum time post-infection evaluated yield shown except for Risch *et al.* (2024) during which two later time points were also investigated (181-200dpi and 201-210dpi) from which no nematodes were recovered.

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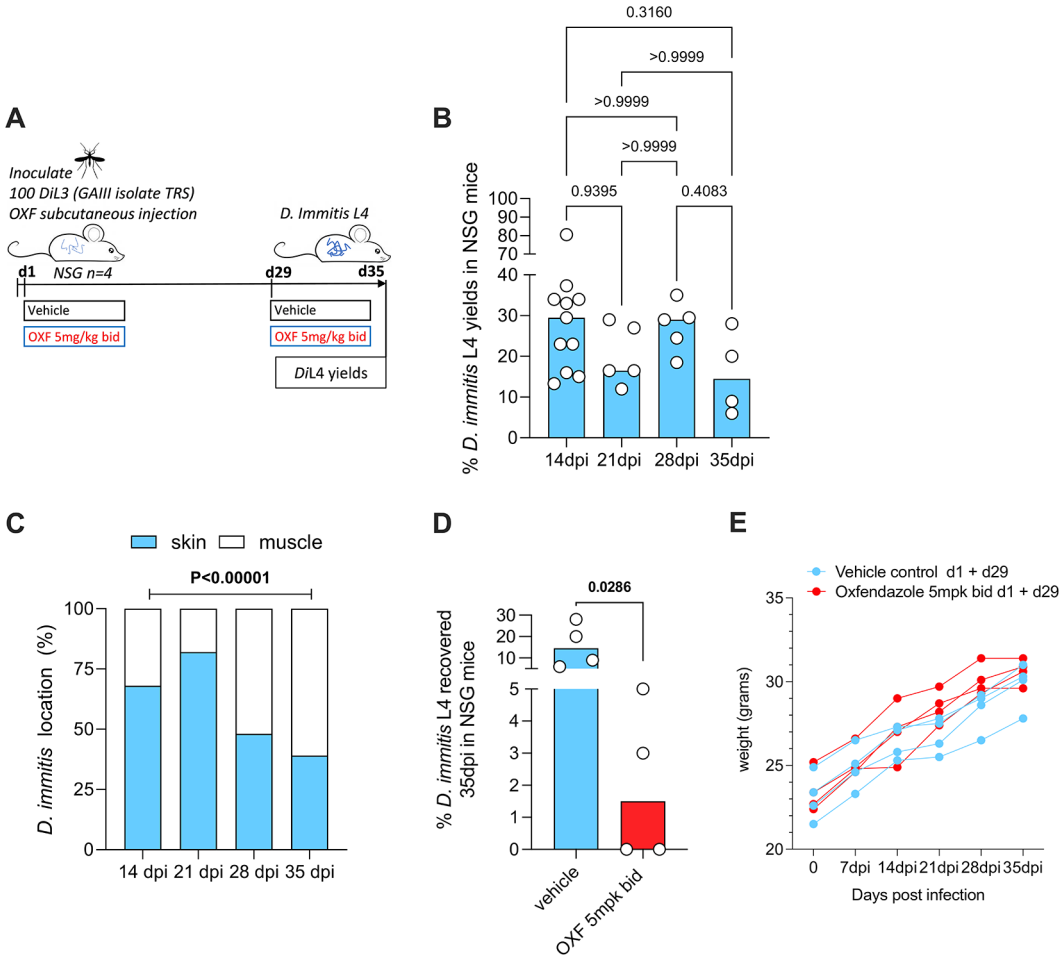

Figure 1. Schematic of experimental design (A). *D. immitis* L4 recovered from NSG mice 2-5 weeks post infection, *Di*L3 expressed as % of initial inoculate (B). Average proportions of L4 larvae in skin/subcutaneous tissue vs muscle (C). Recoveries following oral dosing with bi-daily (bid) oxfendazole (d1+d29) (5 mg/kg), or vehicle control (D). Weight changes in individual mice (E). Bars are median recoveries (B,D) or mean proportions (C) from a single experimental group of 4-5 mice or two-independent experiments combined (2 week data) with 2-to-4-week data previously published by Marriott *et al.* (2023). Significant differences determined by Kruskal–Wallis one-way ANOVA with Dunn's multiple comparison's tests except (C) where the difference in proportions was tested by 2x4 Chi-Square test for trend. Significant differences ($P \leq 0.05$) are indicated in bold, no data excluded.

P=0.029, Dunn's post-hoc test P=0.024). In B.6 RAG2$^{-/-}$/γc$^{-/-}$ mice, MPA treatment did not significantly bolster yields (median 5%, range 2-22%). MPA treatment did increase the frequency of animals with infection in 4/5 CB.17 SCID mice, although heartworm larval recoveries were low and not significantly different to non-treated animals (median recovery=1.5%, range =0-7%) (Figure 1B). Thus, we summarise that RAG2$^{-/-}$/γc$^{-/-}$ mice are initially validated as an alternative susceptible tissue phase larval heartworm model, without requirement for steroid suppression of residual innate immunity. We summarise all mouse strains tested for *D. immitis* susceptibility in Table 1.

Discussion

Following the success of Marriott *et al.* (2023) and Hess *et al.* (2023) in establishing a validated *D. immitis* immunodeficient NSG/NXG mouse preventative drug screening model, our current study demonstrates the ability of NSG mice to sustain GAIII *D. immitis* infection for up to five weeks post-infection with further evidence of larval migration from the skin and subcutaneous tissues into deeper musculature. This suggests that for the first 35 days, development of heartworm larvae emulates that of within natural hosts, whereby the L4-stage migrates from the subcutaneous space into muscles, penetrates the vasculature and arrives in the heart and lungs after 65-70 days (Orihel, 1961; Supakorndej, McCall and Jun, 1994). Similarities in larval length at 14-35 days post infection in NSG mice: (1.5–1.8 mm, 14d & 3.5-4.0 mm, 35d) Hess *et al.* (2023) and NSG/NXG mice (1.2 – 2.8 mm, 14d) Marriott *et al.* (2023) are aligned with growth of *D. immitis* L4 in

Table 2. Efficacy of anthelmintic regimens against *D. immitis* isolates in tissue-phase heartworm larval infection models.

Drug	Dose model	Regimen[1]	Efficacy (n) time post-infection evaluated			
			MO *Di*L3 (LSTM UK)	GAIII *Di*L3 (TRS USA)	MO *Di*L3 (TJU USA)	JYD-34 *Di*L3 (TJU USA)
Ivermectin[2]	0.005 mg/kg NSG	po qdx3 (d1/15/30)			57% (19) d42	
	0.01 mg/kg NSG	po qdx3 (d1/15/30)			73-76% (26) d42	30% (13) d42
	0.3 mg/kg NSG	po qdx3 (d1/15/30)			87% (21) d42	
	0.5 mg/kg NSG	po qdx3 (d1/15/30)			87% (18) d42	
	1.0 mg/kg NSG	po qdx3 (d1/15/30)			87% (11) d42	
	3.0 mg/kg NSG	po qdx3 (d1/15/30)			87% (6) d42	
Moxidectin[2,3]	0.01 mg/kg NSG	po qdx3 (d1/15/30)			96% (8) d42	88% (8) d42
	2.5 mg/kg NSG	sc qdx1 (d1)	65-80% (3) d14	60, 73, 75% (5) d14,21,28		
	2.5 mg/kg NXG	sc qdx1 (d1)	67-88% (5) d14			
Oxfendazole[4]	5 mg/kg NSG	po bidx2 (d1/29)		90% (4) d35		

[1]po = per oral, sc = subcutaneously, qd = once per day, bid = twice per day.
[2]Mean efficacy compared with vehicle control, reported in Hess *et al.* (2023).
[3]Median efficacy compared with vehicle control, reported in Marriott *et al.* (2023).
[4]Median efficacy compared with vehicle, previously unpublished data.

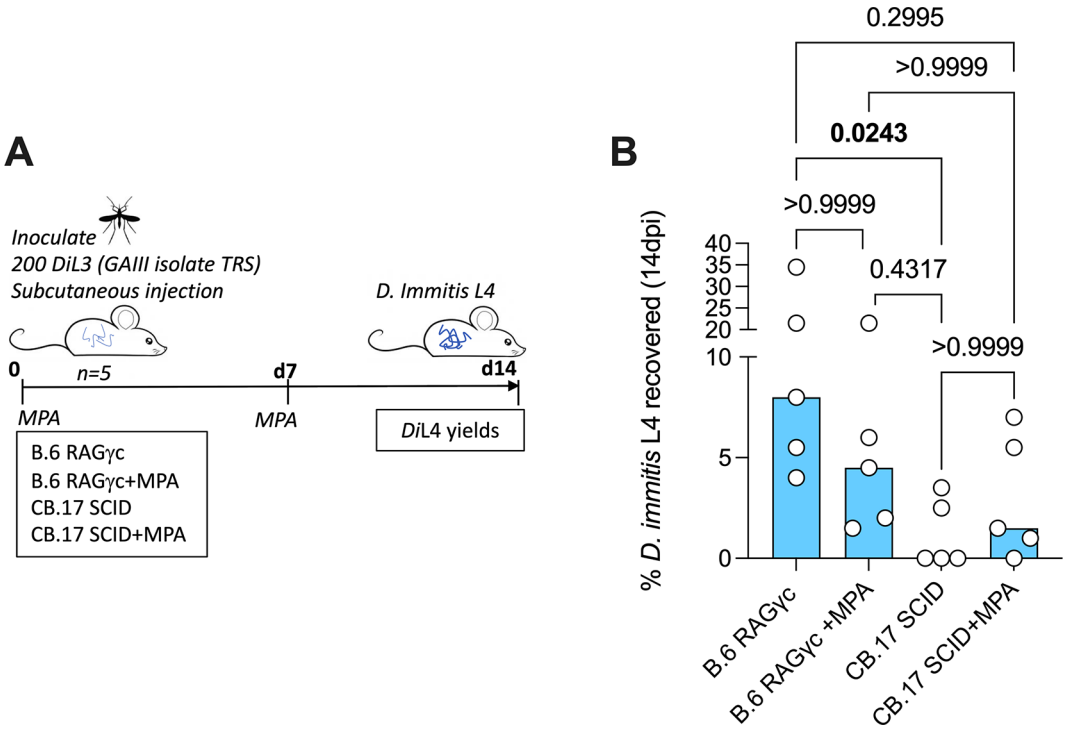

Figure 2. Schematic of experimental design (A). *D. immitis* L4 recovered from B.6 RAG2$^{-/-}$/γc$^{-/-}$ and CB.17 SCID (+/- MPA treatment) 2 weeks post-infection with 200 GAIII *Di*L3 expressed as % of initial inoculate (B). Bars are median recoveries from a single experimental group of 5 mice. Significant differences determined by Kruskal–Wallis one-way ANOVA with Dunn's multiple comparison's tests. Significant differences (P≤0.05) are indicated in bold, no data excluded.

dogs during this timeframe (1.7–2.2 mm, 14d, 3.1-5.6 mm, 30d) (Orihel, 1961; Lichtenfels, Pilitt and Wergin, 1987). Hess and colleagues identified that after 35 days, development of L4 in NSG mice diverged, with retarded growth in murine tissues. Hess *et al.* (2023) also observed no entry of larvae into the heart up to 15 weeks in NSG mice, suggesting L4 larvae may become arrested in development in subcutaneous tissues and muscle after 35 days infection. With the recent data of Risch *et al* (2024) demonstrating MO isolate adult development in B.6 RAG2$^{-/-}$/γc$^{-/-}$ mice, arrested development of late-L4 appears specific to NSG mice, rather than a universal barrier to development in all immunodeficient mice. Regardless of long-term susceptibility, the window of aligned growth in NSG mice is encouragingly sufficient to allow testing of new preventative candidates in this model, utilising regimens emulating once-per-month exposures demanded by current target candidate drug profiles. In prior studies, single-dose injected moxidectin or 3x fortnightly oral ivermectin/moxidectin have been utilised for initial validations (summarised in Table 2). With no regimen demonstrating 100% effectiveness, as seen in dogs, this may indicate an ancillary requirement for host adaptive immune responses to deliver optimum ML efficacy, as prior discussed (Marriot *et al.*, 2023). When we tested oxfendazole in a daily exposure cycle spaced four weeks apart, we demonstrated 90% efficacy, extending validation of the NSG mouse model and demonstrating feasibility of once-per-month drug testing. We selected oxfendazole based on its registered use in companion animals, activity against L3 filarial larvae (Jawahar *et al.*, 2021) and recent demonstrable curative activity against *Litomosoides sigmondontis* infection models (Hubner *et al.*, 2020; Jawahar *et al.*, 2021). Oxfendazole may thus have the potential to be used as an alternative or in combination with ivermectin for monthly oral prevention of infection with drug-resistant *D. immitis* and should be scrutinised for dose-dependent efficacy against resistant isolates.

We explored additional laboratory inbred strains of mice and effects of MPA treatment. C57BL/6J, NOD (NOD/ShiLt), B.6 SCID, and NOD.SCID mouse strains previously investigated by Hess *et al.* (2023) were determined refractory to MO isolate *D. immitis* (summarised in Table 1). Here we identify C.B-17 SCID and B.6 RAG2$^{-/-}$/γc$^{-/-}$ mice as susceptible to *D. immitis* GAIII isolate survival and growth over 14-days. MPA treatments were not necessary for susceptibility in B.6 RAG2$^{-/-}$/γc$^{-/-}$ mice, simplifying onward use for drug testing and avoiding potential drug-drug interactions. MPA treatments were successful in increasing the infection success of CB.17 SCID mice, indicating that inherent immune traits varying between these different genetic backgrounds combined with lymphopenia and deficiency in IL-2/7 receptor signalling dictates early immune control of *D. immitis* larvae in mice. We and others have established both innate (natural killer cell, alternatively activated tissue resident macrophage) and adaptive (IL-4/5/13 producing CD4^{+} T

cell) immune responses combine to orchestrate eosinophil-dependent immune response to developing *B. malayi* larvae in mice (Turner *et al.*, 2018; Pionnier *et al.*, 2020, 2022). Additionally, while this report was under review, Risch *et al.* (2024) published their evaluation of B.6 RAG2 $^{-/-}$/γc $^{-/-}$ mice, demonstrating migration and long-term development of adult nematodes within the heart and lung vasculature (summarised in Table 1). However, some mice developed severe caval syndrome during these later stages of infection, and from a welfare aspect, it would be advisable to limit drug screening endpoints to the late-L4 tissue stage of infection which we have evaluated as a mild procedure. The variety of biological, pharmacological, and genetic modification tools in laboratory mice (many of which available on BALB/c or B.6 inbred backgrounds) may now be applied to pinpoint the basis of immunity against dog heartworm, potentially supporting rational vaccine design. Despite the presence of a patent pending or awarded, in Europe and other territories, (abandoned in USA), for use of mouse models, claims are restricted to the NSG mouse model applied to prophylactic anthelmintic drug screening (Abraham *et al.*, 2028). We therefore suggest that establishment of susceptibility in a variety of alternative mouse models summarised here will allow for unfettered access by the parasitology community for both basic and translational research.

One potential limitation of this new data is that power calculations (n=3) assumed a normal distribution whereas parasite yields were aggregated, requiring non-parametric testing. Despite a potential underpower, due to the potency of oxfendazole being >70% efficacy, and our inclusion of an extra animal per group, this did not affect determining a significant outcome. In future studies, researchers should be wary of aggregated distributions when determining sample size.

Current regulatory requirements demand 100% prophylactic activity in cats or dogs for registration of new heartworm preventatives, meaning it is not currently possible to completely avoid experimental use of cats and dogs. However, with a variety of susceptible mouse strains available (summarised in Table 1), some without current commercial use restrictions, we envisage these new models may become widely adopted by both academic, not-for-profit, and commercial organisations to produce L4 for *in vitro* drug titration evaluations and for initial triaging of compounds and exposure-regimen selections *in vivo*. Future adoption of immunodeficient mice as an initial frontline screen, with short timeframes and without notable impacts on weight or welfare changes arising during the tissue-phase infection period, has the potential to reduce the overall use-requirement of cats and dogs in experimental heartworm research by at least 50%.

Ethics statement

Male NSG, CB.17 SCID and C57BL/6 RAG2$^{-/-}$γc$^{-/-}$ mice were group housed at TRS Labs. within filter-top cages. Animals had continuous access to fresh sterile food, water, and enrichment throughout experiments. Weight was monitored weekly and welfare behaviour monitored daily. Humane endpoints were defined as >20% weight loss and/or observation of adverse behavioural changes which did not improve over a 6h observation period following any remedial treatment by study veterinarian including but not limited to: loss of mobility, starring coat, eye squint, pinched nose, ears pulled back, and/or laboured breathing. Studies were conducted in the USA and approved by the TRS Institutional Animal Care and Use Committee. Protocols were identical to prior approved studies conducted in the UK, approved in the UK by LSTM & University of Liverpool Animal Welfare and Ethics Review Boards and licensed by The UK Home Office Animals in Science Regulation Unit. The manuscript was written in adherence with the ARRIVE 2.0 guidelines.

Data availability

Underlying data

Figshare: Current status of immunodeficient mouse models as substitutes to reduce cat and dog use in heartworm preclinical research, https://doi.org/10.6084/m9.figshare.25250101.v1 (Dagley *et al.*, 2024).

This project contains the following underlying data:

- Supplementary data file_NC3Rs.xlsx

Reporting guidelines

Figshare: ARRIVE checklist, https://doi.org/10.6084/m9.figshare.25690062.v1 (Dagley, 2024).

- ARRIVE NC3Rs_.pdf.

Acknowledgments

We gratefully acknowledge the NIH/NIAID Filariasis Research Resource Center (www.filariasiscenter.org) for maintenance and donation of *D. immitis*.

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Open Peer Review

Current Peer Review Status: ✔ ✔ ✔ ✔

Version 1

Reviewer Report 05 August 2024

https://doi.org/10.5256/f1000research.164362.r299372

✔ **Jan Šlapeta**
[1] The University of Sydney, New South Wales, Australia
[2] The University of Sydney, New South Wales, Australia

Authors demonstrate recovery of *D. immitis* larvae from B.6 RAG2-/-/γc-/- mice without the need for methylprednisolone, thus simplifying onward use of mice for drug testing against *D. immitis* (Di).

Oxfendazole is poorly soluble, would authors have data on Tmax and elimination halflife in mice and potentially in dogs? Generally, I would expect it will be rather quick as far as Tmax and half-life will be likely <12h. This information should be considered when discussing your results and the reality of oxfendazole use in dogs. I assume it was because you chosen "bid" dose to counter the rapid Tmax and elimination. In this context it would have been beneficial to measure the concentration and bioavailability at some key timepoints post dosing. The regimen chose with Di infection got me thinking why was infection at time point zero (day 1) chosen? More realistic dosing would have been only at 28-30 dpi, thus considering all the <30 days larvae.

Could you elaborate on reasoning to increase to 200 Di L3 in some of your experiment? Would 200 L3 be more optimal for future experiments in general to increase the number of detectable larvae at the end of the experiment / power analysis?

Fig 1D / same for Fig 2 B – I don't think the % should be there, you're reporting real values and an arithmetic mean? Please check. It maybe or not maybe % but depends on the starting # either 100 or 200 L3 inoculated.

I am not sure how you calculated your >90% +reduction (results section first paragraph). Please provide the calculation and values used.

Authors should reconsider the introductory sentence (and later in the 3rd paragraph); potentially misleading if they start with felids/cats. It is canids that are the principal and natural vertebrate/mammal hosts esp. domestic dog in this context where the disease and suffering is pronounced and requires intervention. Felids are in host situation just an aberrant host as with

rare development of sexually mature L1 producing Di. I am personally not aware of the term "abbreviated" zoonotic infection. I fully acknowledge the presence of coin lesions in humans, but it is potentially misleading to focus on humans and so zoonosis when the real suffering and almost solely burden in dogs.

In the second paragraphs authors note 60-days, I personally would be extremely cautious about using such number. The registration, most if not all, claim 28/30 days. The research reports cannot be considered as claims and so if any veterinarian would recommend 60 days it would be solely off-label use. I would exercise caution and suggest authors align with the registration claims. No need to go into detail re different MLs and if there is potential or not based on research articles.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Veterinary and molecular parasitologist

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 24 July 2024

https://doi.org/10.5256/f1000research.164362.r299367

Qian Han
[1] Hainan University, Haikou, Hainan, China

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The use of mouse models in heartworm drug screening research holds great significance. It offers an ethical alternative to the experimental use of cats and dogs, reducing the reliance on these companion animals for preclinical drug testing and minimizing potential harm and discomfort to them. Mouse models provide a controlled environment that allows for precise manipulation and monitoring of variables. This enables researchers to systematically study the efficacy, mechanism of action, and potential side effects of different heartworm drugs in a more standardized and reproducible manner. Moreover, the development of mouse models helps in understanding the immune responses and pathophysiological processes underlying heartworm infection and drug treatment. This fundamental knowledge can guide the development of more effective and targeted therapeutics in the future. In conclusion, mouse models are a valuable tool in heartworm drug screening research, accelerating the drug development process and contributing to the fight against this important veterinary parasitic disease.

The presented research provides a valuable contribution to the field of chemoprophylactic prevention of veterinary heartworm disease via the exploration of immunodeficient mouse models in drug screening. The comprehensive comparison of different mouse models, worm isolates, anti-inflammatory treatments, and anthelmintic offers a detailed analysis that strengthens the reliability and validity of the proposed screening models. The addition of new data on the treatment response to oxfendazole and the evaluation of susceptibility in specific mouse strains adds to the novelty and practical application of the study.

However, according to the information of author's current and previous studies, most heartworm larvae are in the tissues, such as muscle and skin, instead of blood vessels. The differences of worm distributions, and drug distribution, metabolism, and excretion in different animals may lead differences in drug efficacy. Authors may consider pharmacokinetic evaluations in combination with drug screening for the interpretation of screening results.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: veterinary parasitology, vector borne pathogens

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 19 July 2024

https://doi.org/10.5256/f1000research.164362.r299364

Timothy G Geary
[1] McGill University, Montreal, Québec, Canada
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This is a valuable addition to the burgeoning literature in this area and addresses an important topic. It is well-written, clear and concise. I have a few points for the authors to consider.

First, two additional recent reports perhaps should be cited. One(Mizuseki M, et al., 2024 [Ref-1]) is perhaps slightly less relevant, but the other (Risch F, et al., 2024 [Ref-2]) should be cited and discussed, as it reports the development of adult stages in mice. I understand that the field moves quickly, but the value of the current manuscript will be enhanced if these papers are included. Two, macrocyclic lactones were less potent and less efficacious in the mouse strains compared to dogs. These drugs are believed to enlist the host immune response in vivo, which obviously may be compromised in these host strains. Some additional discussion around this issue is warranted. Finally, I believe the authors should acknowledge that intellectual property has been filed on at least one of the models (and the claims may be sufficiently broad to cover the others). I believe that patent may have been granted in the EU (at least). This is relevant because it may block drug screening by others. I think it is essential for the authors to discuss this. In addition, a granted patent covers the use of immunosuppressed rats as hosts for this parasite. I believe patents are citable, and this one may also cover ore broadly the use of immunocompromised rodents for drug screening for heartworm preventatives (the authors should check). Interestingly, effects of macrocyclic lactones in these rates closely resemble those in dogs (potency and efficacy), an interesting difference from the mouse that really should be discussed in the current manuscript.

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Is the work clearly and accurately presented and does it cite the current literature?
No

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Anthelmintic pharmacology (basic, clinical, discovery, development, resistance); molecular basis for the host-parasite interaction

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 29 Jul 2024

Jessica Dagley

Thank you for your feedback and comments. We hope we have addressed your points in the updated version and have some comments below:

- While this article was under review, the report by Risch F et al., 2024 was published. We agree this is an important article, which corroborates our data herein, and indicates independent verification of susceptibility to D. immitis isolates in B.6 RAG2-/-gc mice. Importantly this research also demonstrates that there is no biological barrier to the migration and development of adults within the heart and lung vasculature in mice. We have added to data and discussion highlighting this research within our article.

- We believe the Mizuseki M et al., 2024 report to be less relevant to our report, as it details a microfilarial-specific infusion model, and thus do not incorporate into comparisons within this specific brief report.

- While the discussion around the mode of action of MLs is warranted, we have discussed this in much greater detail within an earlier publication (Marriott et al., 2023). We have therefore referenced this, but due to this being a brief report, will not discuss in greater

detail within the text.

- We appreciate the point raised regarding the rat patent; however, we do not deem it appropriate to discuss data within patents filed where data is not yet peer reviewed and published.

- Regarding the mouse patent filed by Boehringer Ingelheim and awarded in Europe (abandoned in USA) based on the work of Hess et al., 2023, we suggest the patent claims are restricted to the NSG model, and a number of emerging alternative immunodeficient mouse lines proven to be susceptible to D. immitis infection should be available for use without restriction in both academia and industry. We have added some discussion regarding this into the main body of text.

Competing Interests: No competing interests were disclosed.

Reviewer Report 19 July 2024

https://doi.org/10.5256/f1000research.164362.r299366

Constantin Constantinoiu
[1] James Cook University, Townsville City, Queensland, Australia
[2] James Cook University, Townsville City, Queensland, Australia

Current status of immunodeficient mouse models as substitutes to reduce cat and dog use in heartworm clinical research

The paper summarises previous research on the use of mice as models of infection with *Dirofilaria immitis* and furthers extends the previous research by demonstrating that:

- GAIII isolates of *D. immitis* can develop up to 5 weeks in NSG mice and that they are susceptible to the action of Oxfendazole;
- GAIII isolate of *D. immitis* can develop up to 2 weeks in RAG2 mice with requirement for suppression of the residual immunity by MPA.

The paper is well written and it presents the data in a clear manner. The only reservation I have is related to the seemingly low number of animals per treatment.

Minor comments/suggestions

Abstract
'Chemoprophylactic prevention' seems to be a pleonasm (prophylaxis means prevention);
Is the word 'veterinary' before 'heartworm disease' needed?
'with or without use of anti-inflammatory treatments' instead of 'following use of anti-

inflammatory treatments'?
'efficacies achieved using different ….' instead of 'efficacies achieved against different ….'?
'evaluation of *D. immitis* development in …' instead of 'evaluation of *D. immitis* susceptibility in …'?

Keywords
Anthelmintics are antiparasitic drugs, do you need both keywords?

Research highlights
What do the authors mean by 'chronic procedures'? Is it the fact that in dogs heartworm disease commonly runs a chronic course? How did the authors get the '67%' value?

Introduction
It seems that some sentences need a little reformulation for their meaning to become clear:

- Humans are at risk of developing abbreviated zoonotic infections causing pulmonary lesions, with increasing reported incidence (Reddy, 2013), frequently confused with tumours (Saha et al., 2022).
- Melarsomine is not registered for use in cats and risks severe adverse events in dogs, requiring complex protracted veterinary clinical case management, exercise restriction and supplementary treatments.

It might be useful to provide a reference for the high efficacy of MLs against the 60 days old larvae.
What do the authors mean by 'do not significantly vary up to 5 weeks of infection'? The infections with *D. immitis* GAIII isolate in NSG mice do not vary under what aspects?

Methods
Animal infections and methods
Were the control mice in the Oxfendazole trial treated with the vehicle subcutaneously (there is no mention in the text and on Fig. 1 it is stated 'Vehicle sc')?

Discussion
'we demonstrated 90% efficacy' instead of 'we demonstrate 90% efficacy'?
'as an alternative or in combination with ivermectin' instead of 'as an alternative or combination with ivermectin'?
'for monthly oral prevention of infection with drug-resistant …' instead of 'for monthly oral treatment of drug-resistant ..'?
'previously investigated' instead of 'prior investigated'?
'eosinophil-dependent immune response' instead of 'eosinophil-dependent immunity'?
'avoid experimental use of dogs and cats' instead of 'avoid experimental use'?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Veterinary parasitology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 29 Jul 2024

Jessica Dagley

Thank you for your feedback. We appreciate the time it has taken to review this article and we take onboard your comments in the updated version.
Regarding estimates of reduction in cat and dog use for drug screening, whilst it is impossible to precisely define the percentage reduction with each drug development programme situation and methodology varying, we considered one standard approach, generating murine pharmacokinetic (PK) data combined with an in silico prediction of efficacy based on the relationship between PK and in vitro determined pharmacodynamics (PD) using D. immitis L4 generated in mice. This will reduce numbers of animals required for empirical testing in vivo by selecting only compounds predicted to mediate threshold effects and calibrate doses in vivo to negate equivocal efficacy readouts. In tandem, testing empirical efficacy in the new mouse infection model will further reduce the use of cats or dogs, thereby reducing the risk of adverse reaction occurrence in these highly sentient species. In a scenario where traditionally 10 experimental drug regimens would be run in long-term, 6-month dog studies (72 dogs), with potential welfare consequences, this new approach would cut the number to 24 dogs (67% reduction) replaced by short-term, mild procedures in an estimated 48 mice.

Competing Interests: No competing interests were disclosed.