










RESEARCH ARTICLE

REVISED Intestinal inflammation and increased intestinal permeability in *Plasmodium chabaudi* AS infected mice

[version 2; peer review: 2 approved]

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Abstract


Background: Gastrointestinal symptoms are commonly associated with acute *Plasmodium spp* infection. Malaria-associated enteritis may provide an opportunity for enteric pathogens to breach the intestinal mucosa, resulting in life-threatening systemic infections.


Methods: To investigate whether intestinal pathology also occurs during infection with a murine model of mild and resolving malaria, C57BL/6J mice were inoculated with recently mosquito-transmitted *Plasmodium chabaudi* AS. At schizogony, intestinal tissues were collected for quantification and localisation of immune mediators and malaria parasites, by PCR and immunohistochemistry. Inflammatory proteins were measured in plasma and faeces and intestinal permeability was assessed by FITC-dextran translocation after oral administration.

Results: Parasitaemia peaked at approx. 1.5% at day 9 and resolved by day 14, with mice experiencing significant and transient anaemia but no weight loss. Plasma IFN γ , TNF α and IL10 were significantly elevated during peak infection and quantitative RT-PCR of the intestine revealed a significant increase in transcripts for *ifng* and *cxc10*. Histological analysis revealed parasites within blood vessels of both the submucosa and intestinal villi and evidence of mild crypt hyperplasia. In faeces, concentrations of the inflammatory marker lactoferrin were significantly raised on days 9 and 11 and FITC-dextran was detected in plasma on days 7 to 14. At day 11, plasma FITC-dextran concentration was significantly positively correlated with

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peripheral parasitemia and faecal lactoferrin concentration.

Conclusions: In summary, using a relevant, attenuated model of malaria, we have found that acute infection is associated with intestinal inflammation and increased intestinal permeability. This model can now be used to explore the mechanisms of parasite-induced intestinal inflammation and to assess the impact of increased intestinal permeability on translocation of enteropathogens.

Keywords

malaria, plasmodium, intestine, permeability, enteritis

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REVISED Amendments from Version 1

We would like to thank the reviewers for their thoughtful comments. We hope the changes have strengthened the manuscript. To summarise, this includes showing pooled data across the multiple experiments. In addition, we have included new data including; villous height and crypt depth (new [Figure 4C](#)), circulating IFN γ has been plotted against *ifn γ* transcript in the colon for 7 and 11 d.p.i. (new [Figure 2D](#)); circulating IFN γ for PcAS-mCherry infections is now shown (new [Figure 5C](#)), and the relationship between circulating IFN γ and permeability is shown for 7, 9 and 11 d.p.i (new [Figure 6B](#)).

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

Introduction

Symptomatic malaria parasite infection is characterised by a cyclical fever, anaemia, and malaise with *Plasmodium spp.*-infected red blood cells (RBCs) detectable in the peripheral circulation. Gastrointestinal symptoms are commonly noted in malaria patients, with diarrhoea reported in both travelers and those residing in malaria-endemic areas (systematically reviewed, ([Sey et al., 2020](#))). For example, diarrhoea was reported in 25% of 451 Ugandan children hospitalised with malaria, significantly more frequently than among malaria-uninfected hospitalised children (11%) ([Lo Vecchio et al., 2021](#)) and treatment with anti-malarial drugs can resolve diarrhoeal symptoms within 48 hours ([Lo Vecchio et al., 2021](#); [Sowunmi et al., 2000](#)), suggesting, but not proving, a causal association.

The primary role of the intestine is digestive, absorbing both water and nutrients whilst creating a barrier to invasion by microorganisms including pathogens. Disturbance of normal intestinal function can result in diarrhoea; a diverse clinical presentation being either watery (e.g. osmotic or secretory) or exudative (i.e. with mucus, blood and cellular discharge). Gastrointestinal pathogens, (protozoal, bacterial and viral) are highly prevalent in malaria-endemic areas and episodes of diarrhoea are common, especially among children ([Troeger et al., 2018](#)). It can, therefore, be challenging to determine whether an episode of diarrhoea is caused by a concurrent malaria infection or is simply coincidental.

One common intestinal pathogen, non-Typhoidal Salmonella (NTS), a particularly frequent cause of invasive bacterial disease (invasive NTS, iNTS) in sub-Saharan Africa resulting in considerable morbidity and mortality (reviewed ([Takem et al., 2014](#))) is, however, significantly more common in people with, or recently recovered from, a clinical episode of malaria than among those with no recent history of malaria infection ([Biggs et al., 2014](#); [Park et al., 2016](#); [Scott et al., 2011](#)). Whilst the features of diarrhoea associated with *Plasmodium* infection remain ill-defined ([Sey et al., 2020](#)), an association with increased intestinal permeability ([Pongponratn et al., 1991](#)) and decreased absorption of vitamin B₁₂ and D-xylose ([Karney & Tong, 1972](#)) has been observed. Furthermore, autopsies of individuals dying from malaria have revealed intestinal haemorrhages ([Dudgeon & Clarke, 1919](#)) and some evidence of

sequestered infected RBCs in villous capillaries ([Pongponratn et al., 1991](#); [Seydel et al., 2006](#)). Given the continuing world-wide burden of clinical malaria ([Organization, 2021](#)), the additional burden of subclinical malaria infections ([Stresman et al., 2020](#)), and the burden of enteropathogenic infections ([Geus et al., 2019](#)), it is important to understand associations between these infections at the intestinal level.

To date, our understanding of malaria-associated intestinal disturbance comes largely from virulent murine models of severe and non-resolving *Plasmodium* infection. For example, *Plasmodium yoelii* infection has been associated with mild caecal inflammation, dysbiosis of the intestinal flora, increased colonisation with *E. coli* and NTS, and increased intestinal permeability (as measured by lactose:mannitol absorption ratios) ([Chau et al., 2013](#); [Mooney et al., 2015](#)). Small intestinal dysbiosis and pathology has also been observed in *Plasmodium berghei* ANKA infected mice ([Shimada et al., 2019](#); [Taniguchi et al., 2015](#)), and traditional serial-blood passaged *Plasmodium chabaudi* infection has been associated with increased cellular influx in the jejunum and increased intestinal permeability ([Alamer et al., 2019](#)). Taking these findings together, a picture is emerging in which severe, acute *Plasmodium spp.* infection in mice induces intestinal inflammation leading to dysbiosis, increased intestinal permeability and increased colonisation by intestinal pathogens. However, the molecular and cellular processes underlying these intestinal responses, particularly whether they are driven by systemic or local inflammation, are unknown.

These murine models of malaria are characterized by high parasitaemia and moderate to severe symptoms, and are therefore less representative of human malaria parasite infections in endemic settings. Therefore, to evaluate the intestinal response in a more physiologically-relevant model of mild to moderate malaria during acute and resolving infection, we have used the recently-transmitted model, in which mosquito transmission attenuates parasite virulence and modifies the host immune response ([Spence et al., 2013](#); [Spence et al., 2015](#)). Moreover, we used a fluorescently-tagged *P. chabaudi* AS line to facilitate imaging of infected RBCs to resolve whether intestinal inflammation is directly associated with parasite sequestration.

Methods**Ethical Statement and mouse procedures at the University of Edinburgh**

This study was reviewed and approved by the Ethical Review Body of the University of Edinburgh. All procedures were carried out in accordance with the UK Home Office regulations (Animals Scientific Procedures Act, 1986) under Project Licence number P04ABDCAA. Throughout this study, all efforts were made to reduce animal usage and ameliorate harm to animals. Mice were housed in the University of Edinburgh Licenced Animal Facilities 60/2605, and all animal procedures were performed in laboratories within the animal facilities. Six to eight weeks old female C57BL/6J mice were purchased from Charles River (Tranent, UK). All animals were maintained

with at least one companion, randomly housed in individually ventilated cages furnished with autoclaved woodchip, fun tunnel and tissue paper at $21 \pm 2^\circ\text{C}$, under a reverse light-dark cycle (light, 19.00 – 07.00; dark, 07.00 – 19.00) at a relative humidity of $55 \pm 10\%$ in a specified pathogen free facility. Mice were housed under these light-dark cycle conditions to allow collection of *P. chabaudi* trophozoites prior to schizogony at 13.00–15.00 hrs, and were allowed to adapt to a reverse-light schedule for at least 7 days before *P. chabaudi* infection. They were fed *ad libitum* an autoclaved dry rodent diet (RM3, Special Diets Services, UK), along with autoclaved water. Animals were monitored according to institutional guidelines, with routine daily health checks and increased monitoring during *P. chabaudi* infection. Euthanasia was performed by cervical dislocation at the end of phenotypic experiments, or by exsanguination under anaesthesia (pentobarbital sodium; Euthatal). This specific method of anaesthesia reduces animal suffering whilst maximising blood volume obtained.

Infection and monitoring

The C57Bl/6 *mus musculus- Plasmodium chabaudi chabaudi* AS animal model of malaria was chosen to minimize host genetic variability and to obtain robust infections with a very low incidence of severe disease. Animals were inoculated i.p. with 1×10^5 PcAS-GFP or PcAS-mCherry-infected RBCs (iRBC) that had been blood passaged 3–6 times since primary infection by mosquito; deemed ‘recently mosquito-transmitted PcAS infection’, as previously described (Spence *et al.*, 2013). GFP or mCherry are constitutively expressed in the cytoplasm of these parasites at all stages of development (Marr *et al.*, 2020).

In total, 208 mice were used in this study, in 8 experiments with groups of 4–7 mice, to provide statistical significance. Mice were infected with GFP-expressing PcAS (4 experiments, $n=79$), mCherry-expressing PcAS (4 experiments, $n=51$), or were uninfected controls ($n=58$). For each experiment, 2 mice were used to expand frozen stocks of stabilate parasites, (16 mice total). Four mice were excluded; three inoculated mice which were uninfected and one with an unexpectedly high parasitemia. For each experimental readout per time-point, two independent experiments were performed.

Mice were weighed and monitored for haemoglobin concentration and parasitaemia by tail snip blood sampling at 18–21hrs of the blood-stage life-cycle for optimal detection of circulating trophozoites, as described previously (Marr *et al.*, 2020). Parasitemia was determined by flow cytometric analysis; diluting 1 μL of tail blood in 1mL of Dulbecco’s phosphate-buffered saline (dPBS, Gibco, UK) containing 5 IU mL⁻¹ heparin sodium (L6510, VWR), and then diluting a further 1:5 prior to acquisition on a BD Fortessa (Becton Dickinson, UK). At least 100,000 events were analysed per sample; gates were set using uninfected control blood using FlowJo V10 (Tree Star), as previously shown (Marr *et al.*, 2020). Processing of control blood was performed prior to that from infected mice to minimise potential cross-contamination upon data acquisition. Haemoglobin concentration (Hb, g/L) was measured using a Hemocue Hb201+ (Radiometer, Sweden).

Weight change was calculated as a proportion of an individual’s pre-infection weight, with measurements taken prior to tail snips. At various days post infection, mice were euthanized (at the time of predicted schizogony) by exsanguination under anaesthesia (pentobarbital sodium; Euthatal) for tissue and/or blood collection following cardiac puncture. Data are shown as ‘day post infection’. As blood sampling was timed to coincide with the presence of circulating trophozoites (i.e. before schizogony, which marks the completion of a replicative cycle) the number of completed replicative cycles is one less than the number of days pi.

Detection of plasma proteins

Cardiac blood was collected into 5 μL of heparin sodium (5 IU mL⁻¹), centrifuged at 10,000g for 5 min, and plasma stored at -70°C for subsequent analysis. For multiplex analysis, a magnetic Luminex assay (LXSAMSM-7, R&D systems, UK) was performed according to the manufacturer’s instructions for IFN γ (BR33), TNF α (BR14), and IL-10 (BR28), using undiluted samples and analysed on a Bio-Plex 200 (Bio-Rad, USA). For IFN γ analysis by enzyme-linked immunosorbent assay (ELISA), plasma was diluted 1:2 and assayed with the mouse IFN γ ELISA MAX deluxe (430804, BioLegend, UK) according to the manufacturer’s instructions. Samples which gave values below the detectable range were reported at the limit of detection for each analyte.

qPCR analysis of mouse intestines

At necropsy, the intestines were divided into five equal lengths (three for the small intestine, two for the large), and cleaned of contents by flushing with dPBS. Tissue was then immersed in 1mL RNAlater (Sigma-Aldrich, UK) and stored at -70°C after chilling according to the manufacturer’s instructions. For isolation of RNA, tissue was transferred to 2mL FastPrep Lysing Matrix D tubes (MP Biomedicals) containing 1mL of TRIzol (Invitrogen). Tissues were then homogenized using a Precellys 24 tissue homogenizer (Bertin instruments) at 30sec on high speed, followed by phenol/chloroform extraction with TRIzol according to the manufacturer’s instructions. Residual DNA was then removed (AM1906, Ambion/Thermo-fisher). Purified RNA was measured using a NanoDrop spectrophotometer and diluted to 100 ng/mL prior to cDNA synthesis using the AffinityScript Multiple Temperature cDNA synthesis kit (200436, Agilent), according to the ‘1st strand cDNA synthesis’ manufacturer’s protocol using 1 μg RNA in a 40 μL volume. For each sample, 2 μL of the cDNA was transferred to a 96-well plate with 18 μL of mastermix (dispensed by robot, Corbett CAS-1200) containing 10 μL Brilliant III Sybrgreen Ultrafast Mastermix (600882, Agilent), 6.4 μL ultrapure water (Gibco), and 0.8 μL of both forward and reverse primers (diluted to 10 μM) for each gene target (sequences listed in Table 1). Samples were run on a CFX96 Real-Time PCR Detection System (Bio-Rad, USA) at 96°C for 3min, followed by 40 cycles of 96°C for 5 sec and 60°C for 10 sec, and data acquisition. Data were analyzed using the comparative threshold cycle (C_t) method. Target gene transcription of each sample was normalized to the respective levels of β -actin mRNA and represented as fold change over gene expression in control animals, as described previously (Lokken *et al.*, 2014). To summarise, to calculate the relative

Table 1. qPCR primers used in the study.

Species	Target	Forward (sense)	Reverse (antisense)
Mus Musculus	<i>Ifng</i>	CAACAGCAAGGCGAAAAAGGATGC	CCCCGAATCAGCAGCGACTCC
Mus Musculus	<i>Cxcl10</i>	GGAICTAAGGGATCCCTCTCG	GGCTCGCAGGGATGATTTCAA
Mus Musculus	<i>Cd68</i>	TGTCTGATCTTGCTAGGACCG	GAGAGTAACGGCCTTTTTGTGA
Mus Musculus	<i>Il10</i>	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT
Mus Musculus	<i>Tnfa</i>	GCAGGTTCTGTCCCTTCACTACTG	TGGAAGCCCCCATCTTTGG
Mus Musculus	<i>Lcn2</i>	ACATTTGTTCCAAGCTCCAGGGC	CATGGCGAACTGGTTGTAGTCCG
Mus Musculus	<i>Cxcl1</i>	GCTTGCTTGACCCTGAAGCTC	TGTTGTGAGAAGCCAGCGTTTAC
Mus Musculus	<i>Mip2</i>	CGCCAGACAGAAGTCATAGCCAC	TCCTTCCAGGTCAGTTAGCCTTGC
Mus Musculus	<i>Il27</i>	TTCCCAATGTTTCCCTGACTTT	AAGTGTGGTAGCGAGGAAGCA
Mus Musculus	<i>bActin</i>	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACTGGCCGT
<i>Plasmodium chabaudi</i>	<i>18S rDNA</i>	AAGCATTAAATAAAGCGAATACATCCTTAT	GGGAGTTTGTTTTGACGTTTATGCG

fold gene expression, an individual reference gene Ct value (β -Actin) is subtracted from the target gene Ct value (Δ Ct), with the mean of control samples then subtracted ($\Delta\Delta$ Ct), and finally the value is taken to two to the negative power ($2^{-\Delta\Delta Ct}$).

Histology and Immunohistochemistry

Rolls of cleaned intestinal tissue were fixed immediately in 10% PBS-buffered formalin, followed by embedding into paraffin prior to sectioning into 4–5 μ m slices onto charged slides. Slides were deparaffinised and rehydrated using an AutoStainer XL (Leica) prior to staining. For visualisation of *Plasmodium* parasites, immunohistochemistry was performed targeting GFP in MT-PcAS-GFP-infected mice. Antigen retrieval was achieved by autoclaving (121°C, 45min) in TRS (pH 6.1; S169984-2, Agilent Dako), followed by washing in PBS/0.1% Tween20. Slides were blocked with 3% hydrogen peroxide for 10min, followed by non-specific horse serum matched to the secondary antibody for 15min, followed by blocking with avidin and biotin for 15min (927301, Bio Legend). Slides were incubated with goat anti-GFP (AF4240, R&D Systems) diluted 1:500 in PBS/0.1%Tween+1%FBS in a humidified chamber at 4°C overnight. After 3 x 15min washes in PBS/0.1%Tween, slides were incubated with biotinylated horse anti-goat IgG H+L (BA-9500, Vector Laboratories) diluted 1:500 for 1h at room temperature. Normal goat IgG (AB-108-AC, R&D Systems) or no primary antibody were used as controls. Finally, slides were stained with DAB (SK-4100, Vector Laboratories) using an ABC reagent kit (32020, Thermo Fisher), according to the manufacturers' instructions with substrate development for 10min, and counterstained with hematoxylin (3136, Sigma Aldrich) using an autostainer XL (Leica).

Villous height and crypt depth were measured on haematoxylin and eosin-stained sections scanned at 40x with a NanoZoomer (Hamamatsu Photonics, Japan) and analysed with QuPath

(v0.2.3) (Bankhead *et al.*, 2017), an open platform for bioimage analysis. Quality assessment scoring (i.e. focus, small artefacts, orientation of the villi) was performed on randomized and blinded scans, followed by collection of 30 measurements of pairs of neighboring crypts and villi using the line tool. Each intestinal roll was divided into three sections (proximal, medial, and distal), with 10 crypts and villi measured in each area. Villus height to crypt depth ratio (Vh:Cd) for each neighbouring pair was calculated, then averaged for either the entire small intestine or each section.

Assessment of intestinal permeability

Intestinal permeability was assessed as described previously (Alamer *et al.*, 2019; Denny *et al.*, 2019; Taniguchi *et al.*, 2015), with modifications. Food was withdrawn from cages for 5 hours prior to oral gavage with 0.1mL of 4-kDa fluorescein isothiocyanate (FITC) dextran (FD4-Ig, Sigma) diluted to 25mg/mL in water, with the time of gavage recorded for each animal. Food was returned after gavage and mice were culled exactly 1 hour post gavage (Volynets *et al.*, 2016; Woting & Blaut, 2018). 100 μ L of plasma (collected as described above) was placed in a black, flat-bottomed 96-well plate, and fluorescence intensity at 520nm measured after excitation at 485nm in a FLUOstar Omega microplate reader (BMG Labtech). FITC-dextran concentrations were calculated from a standard curve of 10-fold serial dilutions of FITC-dextran standard and analysed using Microsoft Excel.

Faecal inflammatory proteins

Large intestines were excised from anus to caecum, split open with scissors and the contents collected with a blunt metal edge into a 2mL eppendorf tube. Samples were placed at -70°C until processing. Contents were weighed and 0.5mL of 'faecal buffer' (0.5% anti-protease cocktail (P8340, Sigma) in dPBS) added. Samples were allowed to rest for 30min at 4°C,

then placed on a vortex adapter for 30min with continuous shaking, as described previously (Fidler *et al.*, 2020). Faecal homogenates were centrifuged at 8,000g for 5min and 250 μ L of supernatant was stored at -70°C. Mouse proteins in faecal supernatants were enumerated by ELISA for IgA (88-50450, ThermoFisher), calprotectin (E1484Mo, Bioassay Technology Laboratory) and lactoferrin (EM1196, FineTest), according to the manufacturer's instructions. Faecal supernatants were diluted 1:2 for lactoferrin detection and 1:400 for IgA, and were undiluted for calprotectin measurements. To measure residual FITC-dextran fluorescence, faecal supernatants were centrifuged a second time at 2,000g for 5min and then diluted 1:4 in water prior to reading at 485/520nm, as outlined above.

Data analysis

Markers of inflammation were compared between mice culled at 4, 7, 11 and 14 days post infection and uninfected control mice. Each post-infection group was compared with the control group using Dunnett's test to account for multiple testing. Where necessary the data were log-transformed to improve symmetry and when there was evidence of heterogeneity in variance between the groups, Dunn's test with Bonferroni adjustment for multiple testing was used instead of Dunnett's test. Correlations between membrane permeability (FITC-Dextran concentration) and parasite load or fecal lactoferrin were assessed using Pearson's correlation coefficient. All statistical

analyses were performed, and graphs made, using GraphPad Prism (v 8.2.1 or v 9.1.0). A p value of <0.05 was considered statistically significant.

Results

In mice infected with blood stage recently mosquito-transmitted *P. chabaudi* AS parasites, expressing GFP (rMT-PcAS-GFP), parasitaemia peaked 9 days post infection (p.i.) at a low to moderate density (median 1.32%, IQR 0.53-4.27, n=51) (Figure 1), in line with expectations (Spence *et al.*, 2013). There were no significant changes in weight compared to uninfected mice, but hemoglobin concentrations declined on day 11 p.i., as observed previously (Marr *et al.*, 2020). There was a clear but very transient inflammatory response on day 7 p.i. (i.e. immediately prior to peak parasitaemia) characterized by raised plasma concentrations of IFN γ and TNF α . Plasma IL-10 concentrations also peaked on day 7 p.i. and were significantly raised for several days. These kinetics are typical of acute malaria parasite infections, denoting a switch from a pro- to anti-inflammatory systemic response to protect the host from immunopathology, driven in part by the co-production of IFN γ + and IL-10+ in activated T cells (Couper *et al.*, 2008a; Couper *et al.*, 2008b; do Rosário *et al.*, 2012).

To determine whether rMT-PcAS-GFP infection and the associated systemic inflammatory response has any intestinal

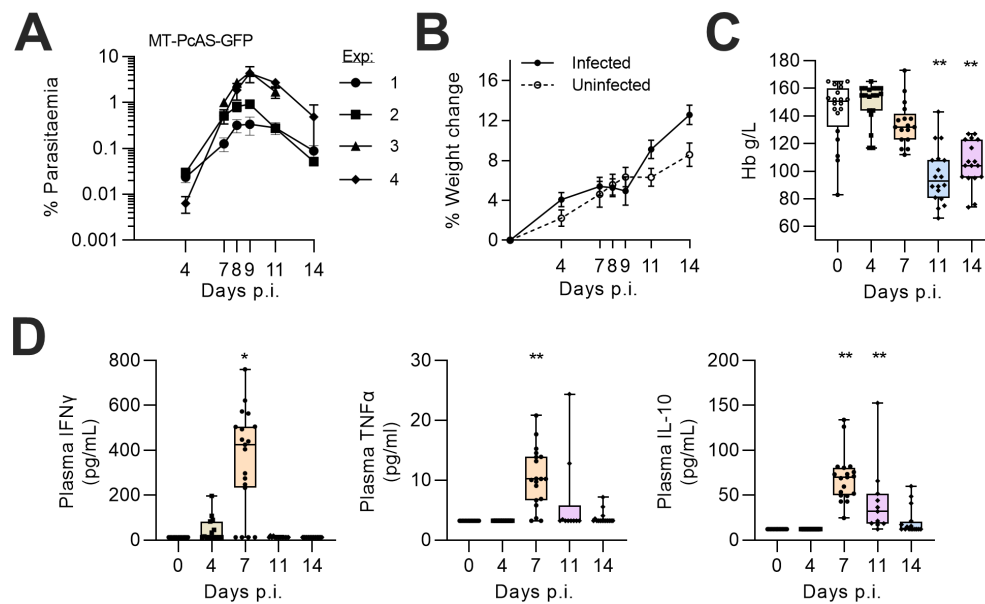


Figure 1. Anaemia and systemic inflammation during *P. chabaudi* infection in mice. Female C57BL/6 mice were infected with 1×10^5 rMT-PcAS-GFP iRBC. **(A)** Peripheral parasitaemia in tail blood was determined by flow cytometry. Data from four replicate experiments, n=4–5 mice per experiment. Data shown as a mean \pm SEM. **(B)** Percentage increase in weight (mean \pm SEM) over time for infected (n=13) or uninfected (n=12) mice. **(C)** Haemoglobin (g/L) from tail blood was determined by haemocue fluorometer from four replicate experiments (n=17–19 per time-point, with 20 controls) with data shown as a box-whisker plot with dots representing individual mice. A significant difference from uninfected mice was determined by ANOVA with Dunnett's test for multiple comparisons on log-transformed data. **(D)** Plasma concentrations of IFN γ , TNF α , and IL-10 determined by Luminex multiplex bead array, with many falling below the limit of detection (12.4 pg/mL, 3.2 pg/mL, and 12.3 pg/mL, respectively). Data shown as box and whisker plot of 10–19 mice per time point. A significant difference from uninfected mice was determined by Dunn's test with Bonferroni adjustment for multiple testing on log-transformed data, with (*) p<0.05, (**) p<0.01. Data pooled from at least three independent experiments, with individual data openly available in extended data files.

consequences, inflammatory markers were analysed in samples of duodenum, jejunum, ileum, caecum, proximal colon and distal colon by qRT-PCR (Figure 2). Intestinal transcript levels for *ifng* and *cxcl10* were raised between 7 and 11 days p.i., and were significantly higher than controls in all sections of the intestine on day 7 p.i. Raised inflammatory markers were particularly evident in the proximal colon with significant elevations of *il10* and *tnfa* on day 7 p.i.; with *lcn2*, *cxcl1*, and *mip2* at 11 days p.i. Whilst plasma IFN γ peaks at 7 days p.i., intestinal transcripts of *ifng* remain elevated in the colon through to day 11 p.i., and show a significant positive correlation

to the circulating protein levels in the proximal colon, but not the distal colon. Thus, *ifng* transcripts can remain in the intestine whilst circulating protein levels have become undetectable. These data are indicative of a generalised, low-grade enteritis which coincides with the period of peak parasitaemia and systemic inflammatory response.

To determine whether parasite localization in the intestine may be driving the enteritis, qPCR for PcAS ribosomal 18S (r18s) was conducted on the same tissue samples (Figure 3). PcAS r18s was detected in all sections of the intestine with the highest

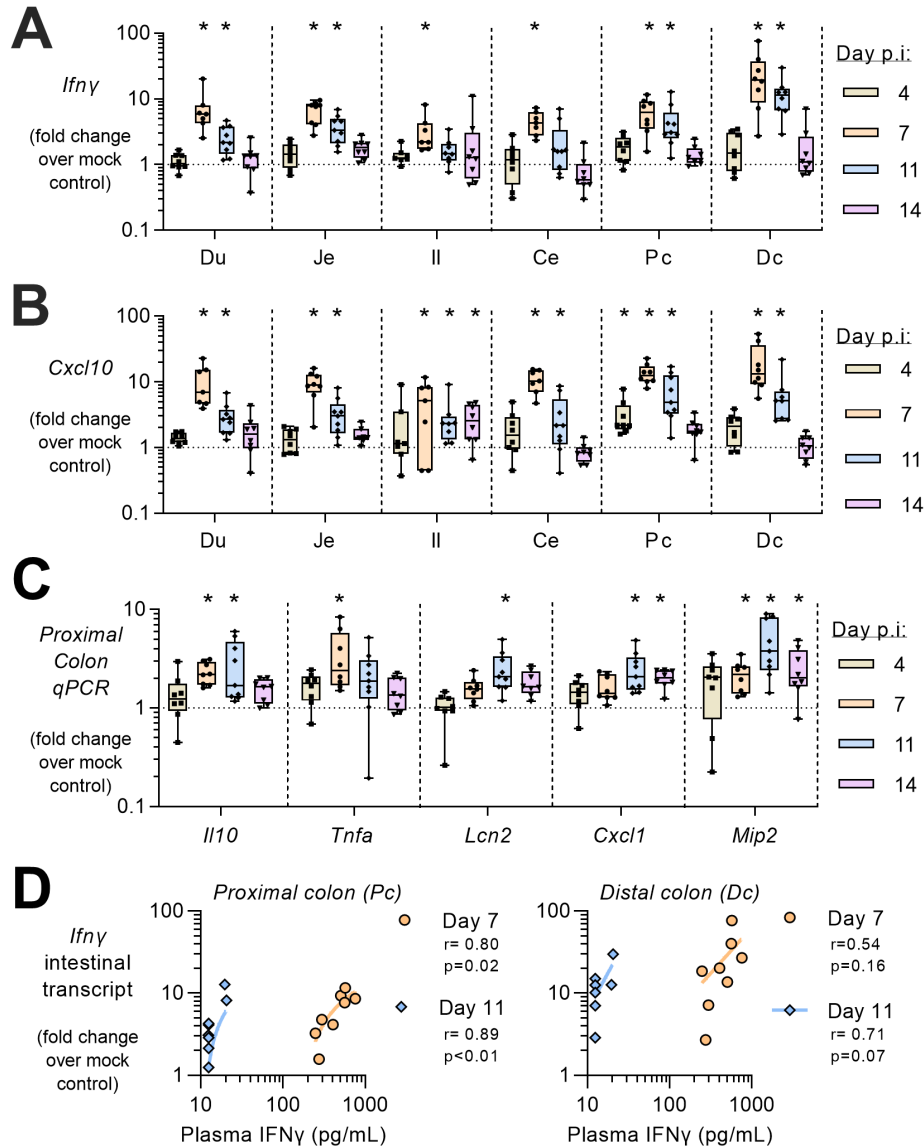


Figure 2. Intestinal inflammation during *P. chabaudi* infection in mice. At 4, 7, 11, and 14 days post infection with MT-PcAS-GFP, mice were culled at schizogony and intestines divided into 6 equal portions; duodenum (Du), jejunum (Je), ileum (Il), cecum (Ce), proximal colon (Pc), and distal colon (Dc). Transcript levels of inflammatory cytokines were determined from 1 μ g RNA by RT-PCR for (A) *ifng*, and (B) *cxcl10* in all tissue sections. (C) In the proximal colon, the levels of *il10*, *tnfa*, *lcn2*, *cxcl1*, *mip2*, and *il27* were also determined. Data shown as fold change over uninfected control mice (with dotted line at 1, mean of n=25) for n=7–8 mice pooled from Experiment 1 and 2 (Figure 1A). Data shown as a box-whisker plot with dots representing individual mice, where a significant difference from uninfected mice was determined by ANOVA with Dunnett's comparison test on log-transformed data, with (*) p<0.05. (D) Correlation analysis from days 7, and 11 p.i. was performed comparing induction of intestinal transcripts of *ifng* in the colon with plasma IFN γ (Figure 1). Data pooled from two independent experiments, n=7–8. Pearson correlation coefficient (r) and p value shown, along with fitted linear regression line. Individual data is openly available in extended data files.

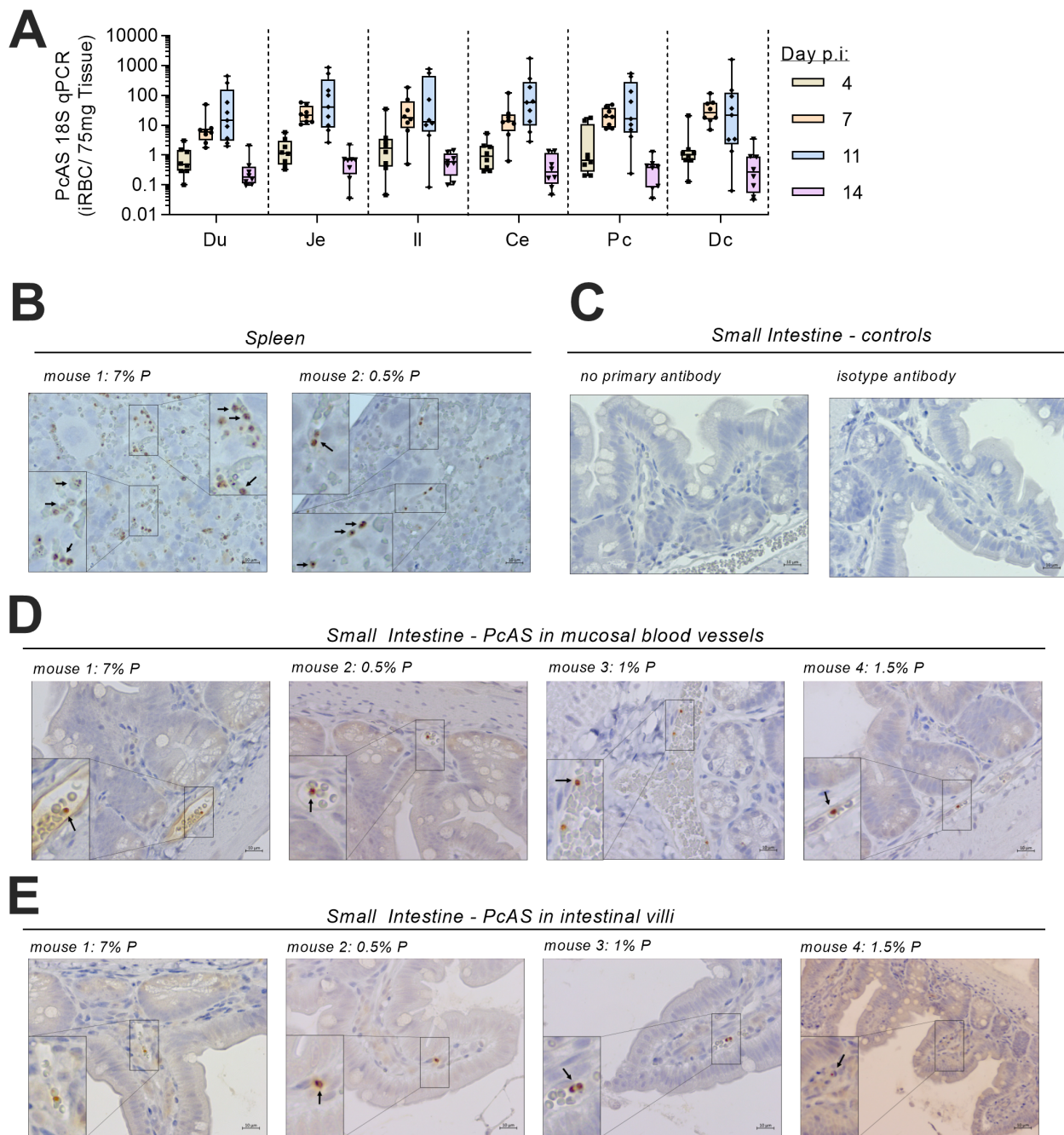


Figure 3. *P. chabaudi*-infected RBCs are present in the mouse intestine. (A) At 4, 7, 11, and 14 days post infection with MT-PcAS-GFP, mice were culled at schizogony and intestines divided into 6 equal portions; duodenum (Du), jejunum (Je), ileum (Il), cecum (Ce), proximal colon (Pc), and distal colon (Dc). Concentrations of *P. chabaudi* r18S were determined by RT-PCR of 1 μ g of tissue RNA run in parallel with a standard of known iRBCs, and then normalised to 75mg of extracted tissue to account for differences between tissue sections. Data pooled from two independent experiments, n=8-9 per time point from Experiment 1 and 2 (Figure 1A). Individual data is openly available in extended data files. (B-E) Immunohistochemistry of formalin-fixed, paraffin embedded intestinal rolls from Experiment 3 (Figure 1A) at 7 d.p.i. (Figure 1A). Tissues were treated with a primary antibody targeting GFP and developed using DAB substrate (brown pigment) with a counterstain of hematoxylin. Representative microscopy images from independent animals from (B) spleen and (C-D) small intestine, with parasites present in blood vessels of the (D) mucosa and (E) villi. Scale bar is 10 μ m. Additional full images, along with scans of slides by nanozoomer, available in extended raw data.

transcript levels detected on days 7 and 11 p.i. Mean intestinal PcAS r18s concentrations were highly correlated with peripheral parasitaemia on both day 7 p.i. ($r=0.84$, $p=0.008$, $n=8$ from 2 independent experiments) and day 11 p.i. ($r=0.99$, $p<0.0001$, $n=9$ from 2 independent experiments).

As mice had not been perfused to remove intravascular blood prior to dissection, it was possible that parasites detected in the intestine were simply circulating in blood. However, we could not rule out the possibility that parasites might be sequestered within blood vessels or had entered the tissues

(Brugat *et al.*, 2014); cytoadherence of *P. chabaudi* AS via binding to the cell-surface receptor ICAM-1 has been reported in the spleen and liver (Cunningham *et al.*, 2017). Therefore, to determine the tissue localization of intestinal rMT-PcAS-GFP, formalin-fixed tissue sections were analysed by immunohistochemistry (Figure 3). Parasites in the spleen were a mixed population of rings, trophozoites and schizonts, as reported previously during schizogony of *P. chabaudi* AS (Brugat *et al.*, 2014). By contrast, in the intestine, only trophozoites and ring forms were seen. Moreover, parasitised red cells in the intestine were clearly confined to intravascular spaces of mucosal blood vessels and smaller villous capillaries. These data suggest that, whilst iRBC circulate freely within intestinal vessels, there is no obvious indication of parasite sequestration in the intestine.

Despite the lack of parasite sequestration in the intestine (Figure 3), evidence of intestinal inflammation (Figure 2) raised the possibility of morphological damage. Although there was no gross intestinal damage (villous epithelium was intact with no pathological evidence of leucocyte infiltration, haemorrhage or necrosis), there was a significant reduction in villous height/crypt depth ratio in the distal small intestine at days 7 and 11 p.i. (Figure 4), indicative of mild villous atrophy and/or crypt hyperplasia (Mills, 2019). However, neither villous height nor crypt depth alone was significantly different in the small intestine.

Given the evidence of a generalized enteritis in PcAS-infected mice, we considered whether there might also be changes in intestinal permeability. For these experiments, mice were infected with recently mosquito-transmitted *P. chabaudi* AS expressing mCherry (rMT-PcAS-mCh) (in order not to interfere with measurement of FITC-dextran). To determine whether any changes in permeability might be linked to intestinal inflammation, faecal inflammatory protein concentrations were also assessed.

Parasitaemia of rMT-PcAS-mCh peaked at a similar density to rMT-PcAS-GFP and between days 8–11 p.i. (median density 1.74%, IQR 0.86-4.67, n=21) (Figure 5). Mice infected with rMT-PcAS-mCh were culled 1 hour after oral gavage with FITC-dextran (Woting & Blaut, 2018), rather than after 4 hours as previously described (Alamer *et al.*, 2019; Denny *et al.*, 2019; Taniguchi *et al.*, 2015). Faecal homogenates (colon contents) were analysed for FITC-dextran, secretory IgA and two biomarkers of intestinal inflammation, calprotectin and lactoferrin; (Lamb & Mansfield, 2011). No significant change was observed for sIgA and calprotectin concentrations. However, faecal lactoferrin concentrations were significantly above baseline on days 7, 9 and 11 p.i., rising 13.7-fold by day 11 (median 79 vs. 1160 pg/gram of colon contents). Furthermore, FITC-dextran concentrations were markedly and significantly lower in the faeces and higher in plasma 1 hour after oral administration on days 7 to 14 p.i. Of note, plasma FITC-dextran concentrations were highly correlated with peripheral parasite densities at day 7 p.i. ($r=0.61$, $p=0.02$) and day 11 p.i. ($r=0.78$, $p=0.02$), with plasma IFN γ at day 9 p.i. ($r=0.80$, $p<0.001$), and with faecal lactoferrin concentrations on days 9 ($r=0.66$, $p=0.05$) and 11 p.i. ($r=0.77$, $p=0.02$)

(Figure 6). Taken together, these data suggest that both intestinal inflammation and increased intestinal permeability are secondary to circulating parasitaemia and associated systemic inflammatory response.

Discussion

Using a rodent model of attenuated, resolving malaria (intraperitoneal injection of recently mosquito transmitted *P. chabaudi* AS) that more closely reflects mild to moderate human malaria infections, with rapidly resolving parasitaemia peaking below 2% and mild to moderate anaemia that resolves upon parasite clearance, we have confirmed previous reports of malaria-associated intestinal inflammation (Alamer *et al.*, 2019; Mooney *et al.*, 2015; Shimada *et al.*, 2019; Taniguchi *et al.*, 2015) and significantly extended those observations. We have shown that parasite-iRBCs circulate freely in the intestinal vasculature but do not appear to sequester in this site; that the enteritis is generalized throughout the small and large intestines and coincident with the development and resolution of parasitaemia; and that intestinal permeability is markedly increased at the peak of parasitaemia and intestinal inflammation. This study has thus established a relevant murine model of malaria-associated enteritis that can be used to further our understanding of malarial disease and enteric co-infections.

Previous studies have looked for sequestration of *Plasmodium spp.*-infected erythrocytes in the intestines. Using a luciferase tagged line of *P. chabaudi* AS, Brugat *et al.* (2014) observed parasites in the liver, spleen and lung but little if any luminescence from whole intestinal tissues (Brugat *et al.*, 2014). *P. falciparum*-infected parasites have been identified in the small intestine, including the intestinal villi, at autopsy (Pongponratn *et al.*, 1991; Seydel *et al.*, 2006) but the resolution of the images was insufficient to determine their precise anatomical localization. In our rMT-PcAS model, immunohistochemistry revealed that ring and trophozoite stage parasites were abundant in the mucosal and villous blood vessels but schizonts were not seen and there was no evidence of cytoadherence of iRBCs to the vascular endothelium or of infiltration of iRBCs into the extravascular spaces or deeper tissues.

Previous studies using murine malaria models that lead to high parasitaemia, severe anaemia and significant weight loss (*P. yoelii ssp* and serially blood-passaged *P. chabaudi*) have reported moderate intestinal inflammation, with infiltration of the intestinal mucosa by monocytes, mast cells, and T cells (Alamer *et al.*, 2019; Chau *et al.*, 2013; Mooney *et al.*, 2015), epithelial damage (Mooney *et al.*, 2015) and villous atrophy and haemorrhages (Taniguchi *et al.*, 2015). By contrast, rMT-PcAS induced enteritis was much more subtle with modestly reduced villous/crypt ratios but no gross epithelial damage, inflammation or haemorrhage. Overall, however, it seems that the severity of malaria-associated enteritis reflects the severity of the infection *per se*. This, taken together with the lack of evidence for sequestration of *P. chabaudi*-parasitised erythrocytes in the intestine and the close temporal correlation between enteritis and circulating parasite density (parasitaemia), suggests that the enteritis may be driven by systemic

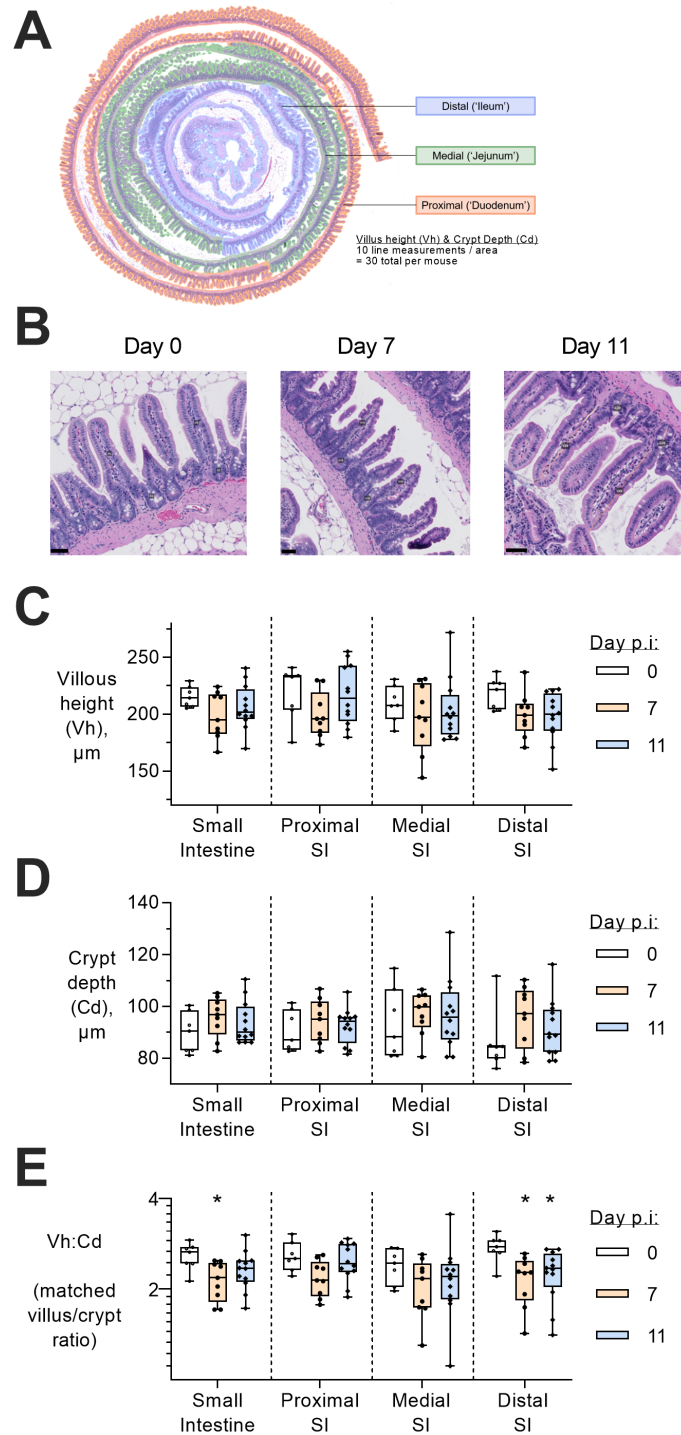


Figure 4. Morphological changes in the small intestine during *P. chabaudi* infection. (A) Small intestinal tissue was rolled and formalin-fixed prior to staining with hematoxylin and eosin. Prepared slides were imaged with a nanozoomer, and subjectively divided into three sections; proximal small intestine (SI) (considered the 'duodenum'), medial SI ('jejunum'), and distal SI ('ileum'). Using blinded images on QuPath software, villus height: crypt depth ratios from 30 pairs of measurements per animal were measured (10 in proximal, distal, and medial), averaged, and displayed by box-whisker plot with dots representing individual mice. (B) Representative images of histology images stained with hematoxylin and eosin, with annotated lines to measure villous height and crypt depth (Day 0 is slide #108, pairs 4 and 5; Day 7 is slide #69, pairs 18 and 19; Day 11 is slide #87, pairs 28 and 29). Scale bar = 50 μm . Entire scans of slides by nanozoomer are available in extended electronic files, annotated with each measurement taken. Line measurements were calculated ($n=10$ per section or $n=30$ for entire small intestine, as above) and then averaged per animal for (C) villous height (Vh, μm) or (D) crypt depth (Cd, μm). (E) Ratios of neighbouring villi height and crypt depth were calculated (Vh:Cd) and then averaged per animal. Data measured from tissues taken from two independent experiments (Experiments 3 and 4, Figure 1A), uninfected ($n=7$), day 7 ($n=9$), and day 11 ($n=12$) post infection. Data shown as a box-whisker plot, with Vh:Cd on log₂ scale, and dots representing individual mice, where a significant difference from uninfected mice was determined by ANOVA with Dunnett's comparison test on log-transformed data, with (*) $p < 0.05$.

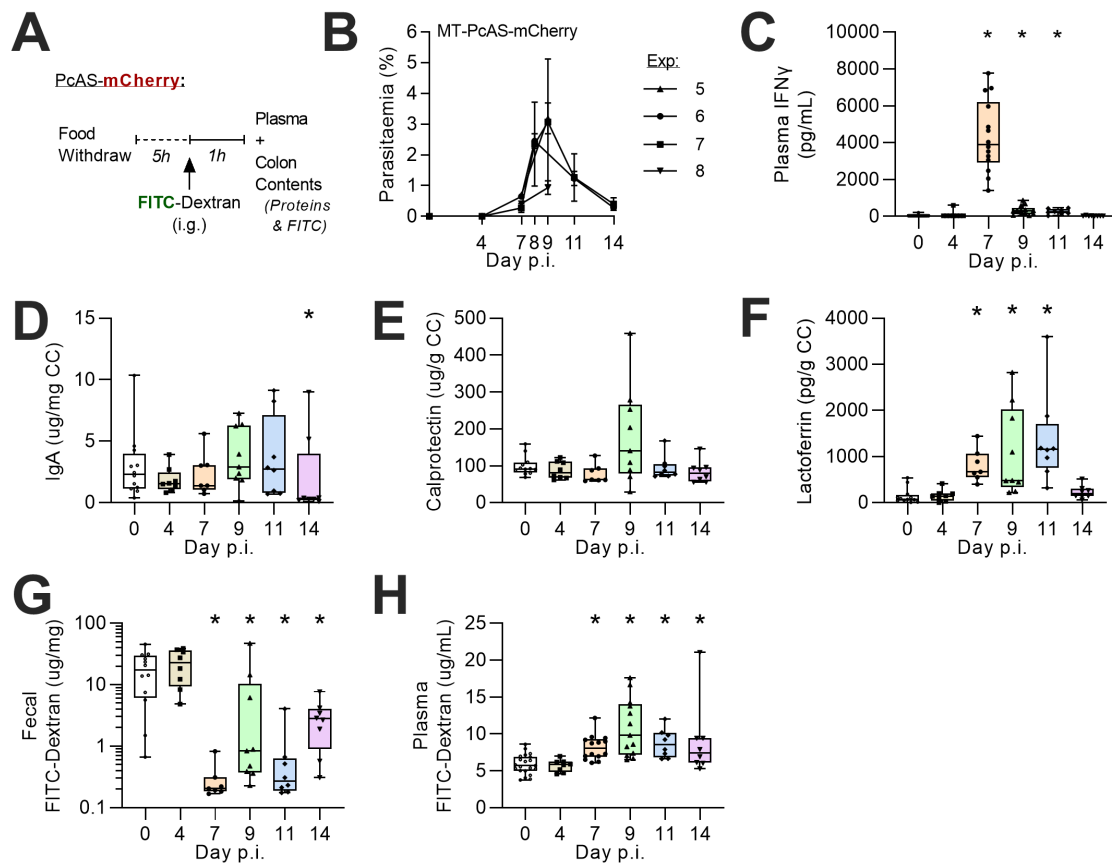


Figure 5. Increased membrane permeability and faecal lactoferrin during *P. chabaudi* infection. Female C57BL/6 mice were infected with 1×10^5 rMT-PcAS-mCh-iRBCs. **(A)** At various time-points, uninfected or rMT-PcAS-mCh infected mice were given FITC-dextran solution by oral gavage and culled 1 hour later. **(B)** Prior to schizogony, peripheral parasitaemia from tail blood from four independent experiments was determined by flow cytometry. Data shown as mean \pm SEM, n=4–5 per experiment. **(C)** Plasma concentrations of IFN γ determined by ELISA, with the limit of detection at (39 pg/mL). Data shown as box and whisker plot of 8–14 mice per time point, with 21 controls, pooled from the four independent experiments. **(D–G)** Colon content (CC) concentrations of **(C)** immunoglobulin A (IgA), **(D)** calprotectin, and **(E)** lactoferrin were determined by ELISA from faecal homogenates. **(G)** Fluorescence of FITC-dextran in faecal homogenates was measured. Data displayed as box-whisker plot with dots representing individual mice, n=7–12. **(H)** Plasma concentrations of FITC-dextran were determined, n=7–12 per time-point with 21 controls. Data pooled from at least two experiments per time point, with additional mice for FITC-dextran **(G)** from pilot data (Experiment #5 in panel 5B). A significant difference from uninfected mice was determined by ANOVA with Dunnett's comparison test on log-transformed data, with (*) p<0.05.

inflammation rather than localization of parasitised RBCs in the intestine.

Although the enteritis observed during rMT-PcAS infection was relatively mild, it was sufficient to cause a marked increase in intestinal permeability during the period of peak parasitaemia, as evidenced by very rapid translocation of FITC-dextran from the gut lumen (colon contents) into the plasma. Moreover, this increased permeability was highly correlated with both parasitaemia and intestinal inflammation (faecal calprotectin), suggesting a causal pathway in which parasitaemia drives systemic inflammation, systemic inflammation drives enteric inflammation and enteric inflammation drives increased intestinal permeability.

Increased intestinal permeability has been demonstrated in humans infected with *P. falciparum* (Pongponratn *et al.*, 1991)

and in *P. yoelii nigeriensis*-infected mice (Chau *et al.*, 2013) (in both cases using the lactulose mannitol test (Fleming *et al.*, 1990)) and in more *P. berghei* ANKA, *Plasmodium yoelii* 17XNL, and PcAS models (using FITC-dextran) (Alamer *et al.*, 2019; Denny *et al.*, 2019; Taniguchi *et al.*, 2015), with varying degrees of increased permeability observed either early or at the peak of infection. The consequences of this change in intestinal permeability, especially in terms of maintenance of the barrier function of the intestinal epithelium and risk of translocation, invasion and systemic dissemination of enteric pathogens such as NTS (Mooney *et al.*, 2019), remain to be fully discerned.

This study reveals novel observational changes in the intestine during a mild, avirulent murine malaria parasite infection. Future work is needed to unpick the mechanism by which intestinal inflammation is induced, and how this relates to

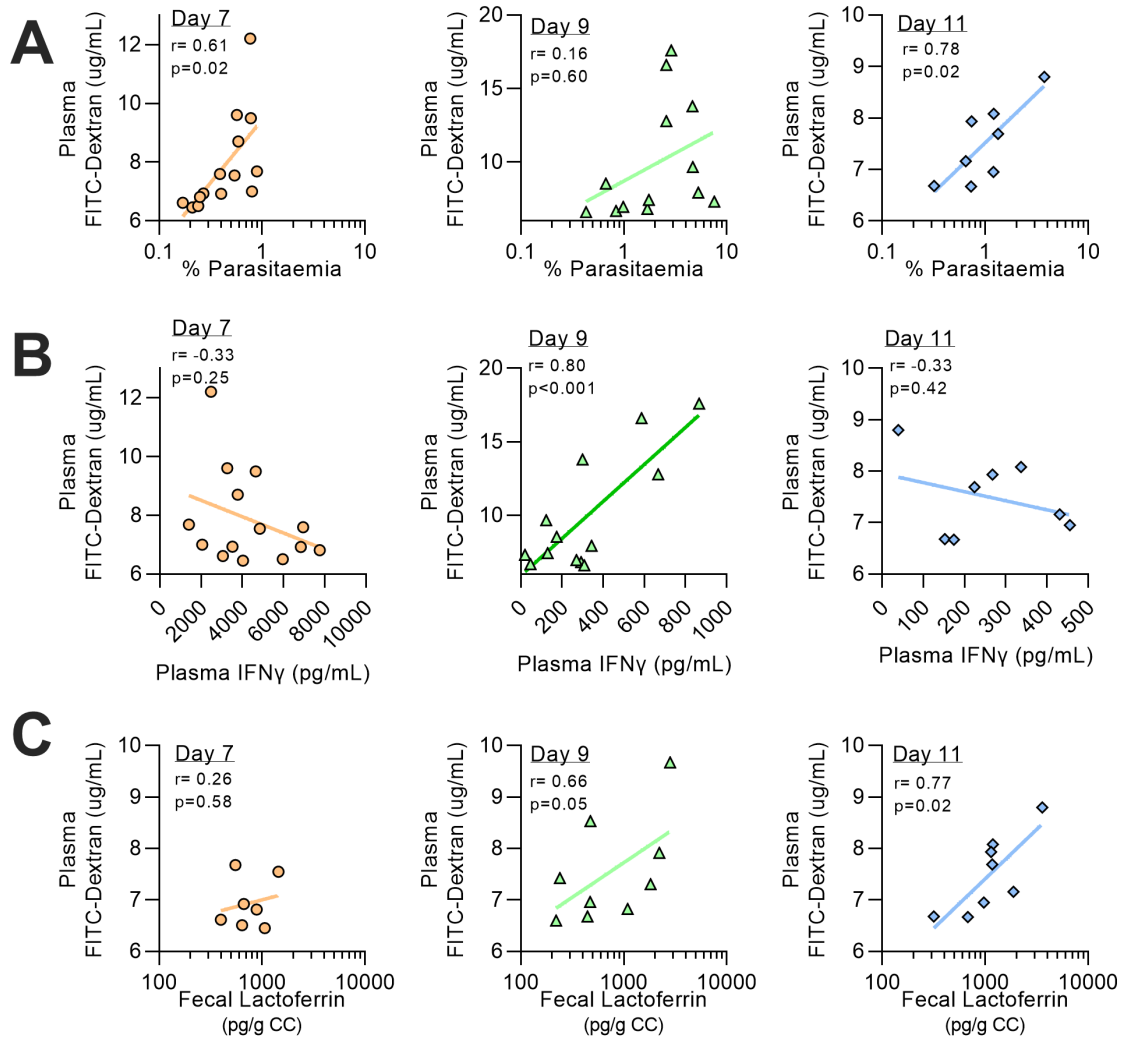


Figure 6. Relationship between parasitemia, faecal inflammation and intestinal permeability. Correlation analysis from mice infected with rMT-PcAS-mCh (Figure 5B) from days 7, 9, and 11 p.i. was performed comparing permeability (plasma FITC-dextran) with (A) peripheral parasitemia (%), prior to schizogony, (B) plasma IFN γ (pg/mL), and (C) faecal lactoferrin levels (pg per g of colon contents, CC). Data combined from 2 experiments per time point, with additional mice for panel (A) at day 7 and 9 from pilot data (Experiment #5 in Figure 5B) (n=7–14 mice per time point). Pearson correlation coefficient (r) and p value shown, along with fitted linear regression line.

intestinal permeability. This would include phenotypical analysis of the cellular source of the inflammatory mediators observed (e.g. IFN γ , TNF α , lactoferrin), as well as their location within the tissue and their contribution to intestinal permeability.

In summary, we have established a clinically relevant murine model of malaria-associated enteritis characterized by systemic and local inflammation and increased intestinal permeability. This model can be exploited to better understand the pathophysiology of enteric disease during malaria infections and to understand the mechanisms by which current or recent malaria infections substantially increase the risk of invasive enteric bacterial infections.

Data availability

Underlying data

Open Science Framework: Malaria in the Murine Intestine, <https://doi.org/10.17605/OSF.IO/EDQTK>. (Mooney, 2022a)

This project contains the following underlying data:

- Methods - Animal Numbers.xlsx
- Fig 1 - Anemia by Hemocue.xlsx
- Fig 1 - Parasitemia_PcAS-GFP.xlsx
- Fig 1 - Plasma Protein by Luminex.xlsx

- Weight Change.xlsx
- Mouse Luminex Raw File.rbx
- Parasitemia FACS Files – Raw and Analysed
- Fig 2 - Inflammation qPCR in Tissue.xlsx
- qPCR Raw Files.xlsx
- 18S PCR Raw Files
- Fig 3 - 18S Plasmodium qPCR in Tissue.xlsx
- Histology Images.jpg
- H'n'E Inventory and Quality Scoring _ Analysed JM 16 Aug.xlsx
- QuPath Project SI.zip
- FACS Raw Files
- Fecal Supe ELISAs.xlsx
- Fecal Supe FITC.xlsx
- FITC-Dextran Plasma RFU.xlsx
- Parasitemia-PcAS mCherry.xlsx
- mCherry correlation data.xlsx

Edinburgh Datashare: Malaria in the Murine Intestine, <https://doi.org/10.7488/ds/3434>. (Mooney, 2022b)

This project contains the following underlying data:

- Figure3-IHC_for_malaria_in_Small_Intestine.zip
- Figure4-HnE_of_Small_Intestine.zip

Reporting guidelines

Open Science Framework: ARRIVE checklist for “Intestinal inflammation and increased intestinal permeability in Plasmodium chabaudi AS infected mice”, <https://doi.org/10.17605/OSF.IO/EDQTK>. (Mooney, 2022a)

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 **Kevin Joseph Maloy** 

School of Infection and Immunity, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK

I thank the authors for having comprehensively addressed my comments and suggestions. The revised manuscript represents a solid addition to this field and I am happy to approve version 2.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Mucosal Immunology

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 12 October 2022

<https://doi.org/10.21956/wellcomeopenres.20398.r52674>

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 **Rahul Vijay** 

¹ Center for Cancer Cell Biology, Immunology and Infection, Rosalind Franklin University, Chicago, IL, USA

² Rosalind Franklin University of Medicine and Science, Chicago, IL, USA

This reviewer does not have any additional comments to the manuscript.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Immunology, 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.

Version 1

Reviewer Report 25 July 2022

<https://doi.org/10.21956/wellcomeopenres.19680.r51410>

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? **Rahul Vijay** 

¹ Center for Cancer Cell Biology, Immunology and Infection, Rosalind Franklin University, Chicago, IL, USA

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In this study the authors report that gastrointestinal symptoms noted during malaria are indeed associated with the malarial disease and are not just coincidental. Using blood stage recently mosquito-transmitted *P. chabaudi* AS, they show the presence of low-grade intestinal inflammation and increased intestinal permeability that is concomitant with peak parasitemia. While parasite sequestration in the intestine is not observed, infected RBCs were seen circulating in the intestinal vasculature.

As noted by the authors similar observations have been made in other mouse models of malaria but these observations, they claim, seem to better represent what is normally observed during human malaria in endemic regions.

Major points:

- While assessing transcripts levels of various cytokines in the intestine, the authors have grouped *il10* along with the inflammatory markers. As the authors may be very well aware, IL10 is considered anti-inflammatory cytokine and hence they need to make that clear in the text. It is also recommended that they give a brief description as to the implications of both pro and anti-inflammatory cytokines upregulated simultaneously.

Minor points:

- The study is merely observational without any mechanistic experimentation. Despite this, the authors have attempted to draw parallels between intestinal complications arising from human malarial disease and those that they observed in this study (using this particular mouse model of malaria), thus validating why their study more representative and useful for experimentation to learn that particular aspect during malaria.

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?

Partly

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?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Immunology, 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, however I have significant reservations, as outlined above.

Author Response 14 Sep 2022

Jason Mooney

In this study the authors report that gastrointestinal symptoms noted during malaria are indeed associated with the malarial disease and are not just coincidental. Using blood stage recently mosquito-transmitted *P. chabaudi* AS, they show the presence of low-grade intestinal inflammation and increased intestinal permeability that is concomitant with peak parasitemia. While parasite sequestration in the intestine is not observed, infected RBCs were seen circulating in the intestinal vasculature.

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RESPONSE:

Thank you for your comments and for this observation. The simultaneous production of both pro- and anti-inflammatory cytokines is well noted, and its purpose is to protect the host from immune-mediated pathology. In fact, previous work from co-author Eleanor Riley and others have shown that, in a highly virulent *P. yoelii* malaria model, Th1 cells are a major source of both IL-10 and IFN γ (Couper et al. 2008, DOI: 10.1371/journal.ppat.1000004). This work was then extended by Rosario et al. (2012) (DOI: 10.4049/jimmunol.1102755) during virulent *P. chabaudi* infection using IL-10 reporter mice. Therefore, we have now included additional clarification in the results section. Thank you for the opportunity for us to provide additional context to this phenomenon for the reader.

Updated text:

Results (paragraph 1):

“These kinetics are typical of acute malaria parasite infections, denoting a switch from a pro- to anti-inflammatory systemic response to protect the host from immunopathology, driven in part by the co-production of IFN γ and IL-10 in activated T cells ((Couper, Blount et al. 2008, Couper, Blount et al. 2008, do Rosário, Lamb et al. 2012)).”

Minor points:

The study is merely observational without any mechanistic experimentation. Despite this, the authors have attempted to draw parallels between intestinal complications arising from human malarial disease and those that they observed in this study (using this particular mouse model of malaria), thus validating why their study more representative and useful for experimentation to learn that particular aspect during malaria.

RESPONSE:

Thank you for your point and we agree this is entirely observational work. We hope to utilize this mouse model, along with extending to human malaria, to address both the clinical relevance and underlying mechanism(s). We have clearly defined this work as observational and further commented on future mechanistic work in the discussion.

Updated text:

Discussion (paragraph 6):

“This study reveals novel observational changes in the intestine during a mild, avirulent murine malaria parasite infection. Future work is needed to unpick the mechanism by which intestinal inflammation is induced, and how this relates to intestinal permeability. This would include phenotypical analysis of the cellular source of the inflammatory mediators observed (e.g. IFN- γ , TNF- α , lactoferrin), as well as their location within the tissue and their contribution to intestinal permeability.”

Competing Interests: None

Reviewer Report 15 June 2022

<https://doi.org/10.21956/wellcomeopenres.19680.r49962>

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Kevin Joseph Maloy

School of Infection and Immunity, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK

This study characterizes intestinal pathology in a well characterized murine model of malaria infection. This is an important topic because human malaria patients often exhibit gastrointestinal symptoms, especially diarrhoea, and they are also more prone to develop invasive disease associated with non-typhoidal Salmonella infection. Determining the contribution of malaria infection versus coincidental enteric infections to intestinal pathology is difficult, so mouse models of malaria are useful in this context. There have been reports of intestinal pathology in mouse models of severe malaria, but the extent of intestinal alterations in milder rodent malaria infections (that mimic the majority of infections in endemic areas) had not been investigated. Hence, this study employed infection with 'recently mosquito-transmitted' *Plasmodium chabaudi* AS (PcAS) and assessed intestinal pathology.

Figure 1 presents data showing that the PcAS infection elicited a consistent pattern of low-to-moderate parasitaemia that peaked around day 9 post-infection (pi). Further analyses of one experiment indicated that there was a transient systemic inflammatory cytokine response on day 7 pi. It would be helpful to know if this pattern of systemic cytokine release was reproducible across multiple experiments.

Figure 2A and B demonstrate that PcAS infection also led to increased expression of *ifng* and *cxc110* along the entire intestinal tract and that these peaked again at day 7 pi. Figure 2C extended the analyses to additional markers for the proximal colon samples. Again, it would be helpful to know that these changes were reproducible across multiple experiments. In addition, whether the changes in gene expression corresponded to increased mediator secretion could have been tested using intestinal explants followed by Luminex and/or ELISA assays of the supernatants.

Figure 4 presents histological analyses of changes in small intestinal morphology in PcAS-infected mice. They observed a modest, transient reduction in villus/crypt ratio on day 7 pi and infer that this reflects 'mild villous atrophy and or crypt hyperplasia'. It should be possible to discern these by separately plotting the villous and crypt lengths. In addition, it would be of interest to know whether the composition and/or activation status of leukocyte populations in the lamina propria was affected by PcAs infection, although this is perhaps a question for future studies.

Figure 5 presents data showing that PcAS infection also led to increased intestinal permeability, as evidenced by increased levels of plasma FITC-dextran, as well as elevated lactoferrin in the colon contents. Figure 6 extends these analyses by showing that these increases correlated with the levels of parasitaemia, suggesting that they are a consequence of the systemic infection. In this respect, it would be of interest to determine whether plasma IFN γ and TNF levels also correlated positively with the measures of intestinal permeability.

Overall, the data are clear, well controlled and very nicely presented. The conclusions made by the investigators are balanced and reasonable and the discussion places them into the wider context and identifies areas for further study.

The key strengths of the study are that it represents a good model of milder malaria infection and presents clear data and detailed kinetic analyses. The model offers scope for further development to better understand the consequences of altered intestinal permeability on the ongoing malaria infection, as well as predisposition to enteric pathogens.

The limitations are that the data are largely descriptive and offer limited mechanistic insight. For example, which cytokines are driving the increased intestinal permeability and what are the key cellular sources of these inflammatory mediators? Are they all systemic or are some local to the intestine? The model provides a solid basis for interrogating these and other questions.

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: Mucosal Immunology

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RESPONSE:

Thank you. This is a very valid point. Previously, we showed data from a representative experiment for clarity in figures 1-4, as the data showed similar conclusions across independent experiments. In response to your comment, and for uniformity, all graphs in this updated manuscript version now show pooled data from at least three independent experiments (*including Fig 2A-C, Fig 3A, and Fig 4*). As with all WOR publications, raw data is open and accessible for full scrutiny, and version 1, with the representative data, remains available online.

Figure 2A and B demonstrate that PcAS infection also led to increased expression of ifng and cxcl10 along the entire intestinal tract and that these peaked again at day 7 pi. Figure 2C extended the analyses to additional markers for the proximal colon samples. Again, it would be helpful to know that these changes were reproducible across multiple experiments. In addition, whether the changes in gene expression corresponded to increased mediator secretion could have been tested using intestinal explants followed by Luminex and/or ELISA assays of the supernatants.

RESPONSE:

(Comment 1) As outlined above, pooled data from two independent experiments is now shown.

(Comment 2) We agree that directly testing for a correlation between mRNA and inflammatory protein expression within tissues, either by explant culture and/or FISH,

would be definitive experiments. Here, we have asked whether circulating IFN γ correlates to intestinal transcripts, and have graphed the transcript level of *Ifny*, in both the proximal and distal colon, against circulating plasma levels in new Figure 2B. At 7 d.p.i, when circulating IFN γ is highest, there is a strong positive correlation. As Th1 cells are a major source of IFN γ in PcAS infection, this isn't unexpected. However, at 11 d.p.i, when circulating IFN γ has significantly diminished, *Ifny* transcripts remain in the colon. This data suggests that IFN γ may come from both local and systemic sources, depending on the infection dynamics and we have discussed this conclusion further in paragraph 1 of the results. Indeed, dissecting out the cellular sources, along with their location within the tissue (i.e. in the blood vessels and/or lamina propria) would be important in future. Thank you for the thoughtful comment.

Updated text for 'Comment 2':

Results (paragraph 2):

"Whilst plasma IFN γ peaks at 7 days p.i., intestinal transcripts of *ifng* remain elevated in the colon through to day 11 p.i., and show a significant positive correlation to the circulating protein levels in the proximal colon, but not the distal colon. Thus, *ifng* transcripts can remain in the intestine whilst circulating protein levels have become undetectable. These data are indicative of a generalised, low-grade enteritis which coincides with the period of peak parasitaemia and systemic inflammatory response."

Figure 4 presents histological analyses of changes in small intestinal morphology in PcAS-infected mice. They observed a modest, transient reduction in villus/crypt ratio on day 7 pi and infer that this reflects 'mild villous atrophy and or crypt hyperplasia'. It should be possible to discern these by separately plotting the villous and crypt lengths. In addition, it would be of interest to know whether the composition and/or activation status of leukocyte populations in the lamina propria was affected by PcAs infection, although this is perhaps a question for future studies.

RESPONSE:

(Comment 1) We originally graphed villous height to crypt depth ratio, as this is a common histomorphologic measurement used on neighbouring villi and crypts. As the reviewer rightly points out, this measurement cannot differentiate between villous atrophy and crypt hyperplasia. In the new Fig 4, we have now graphed solely villous height and crypt depth. While there is a trend for both villous atrophy and crypt hyperplasia, the data is not significant. In our analysis, differences are only observed when neighbouring villi and crypts are compared against one another. This calculation may be more sensitive, as the Vh:Cd ratio takes into account the proliferation of intestinal progenitor/stem cells within the crypt against the blunting of the neighbouring villous rather than 10/30 randomised single measurements. Pathological changes may not be uniform across the intestinal surface and thus larger sampling may be warranted in future studies. Regardless, the data here suggests that neither villous atrophy nor crypt hyperplasia alone is a dominant phenotype. In all, we would conclude that changes in intestinal pathology during PcAS are mild.

Updated text for 'Comment 1':

Results (paragraph 5):

"However, neither villous height nor crypt depth alone was significantly different in the small intestine."

(Comment 2) We agree that understanding the cellular infiltrate would be important. We are actively exploring this avenue in order to fully characterise the source of faecal lactoferrin, which is presumably via an influx of intestinal neutrophils. These points have been expanded on in a new paragraph in the discussion.

Updated text for 'Comment 2':

Discussion (paragraph 6):

"This study reveals novel observational changes in the intestine during a mild, avirulent murine malaria parasite infection. Future work is needed to unpick the mechanism by which intestinal inflammation is induced, and how this relates to intestinal permeability. This would include phenotypical analysis of the cellular source of the inflammatory mediators observed (e.g. IFN- γ , TNF- α , lactoferrin), as well as their location within the tissue and their contribution to intestinal permeability."

Figure 5 presents data showing that PcAS infection also led to increased intestinal permeability, as evidenced by increased levels of plasma FITC-dextran, as well as elevated lactoferrin in the colon contents. Figure 6 extends these analyses by showing that these increases correlated with the levels of parasitaemia, suggesting that they are a consequence of the systemic infection. In this respect, it would be of interest to determine whether plasma IFN γ and TNF levels also correlated positively with the measures of intestinal permeability.

RESPONSE:

We agree, and appreciate that it would be useful to show all parameters in a single figure for related animals. Therefore, we now show circulating IFN γ (measured by ELISA) in Figure 5 and, in Figure 6B, we have plotted this plasma IFN γ against permeability (FITC-Dextran). The data shows that a positive correlation is found only at 9 d.p.i., when IFN γ has diminished. Nevertheless, it is important to recognise that the link between IFN γ and intestinal permeability may not be linear and as the reviewer suggests, it may be important to measure additional inflammatory mediators, such as TNF. We have included additional comments in the discussion (paragraph 6, pasted above), highlighting that a more robust characterisation of proteins pertinent to intestinal permeability, rather than indicative of a generalised inflammatory response such as IFN γ , would be of great interest. We thank the reviewer for his comment and look forward to future research on this topic.

Overall, the data are clear, well controlled and very nicely presented. The conclusions made by the investigators are balanced and reasonable and the discussion places them into the

wider context and identifies areas for further study.

The key strengths of the study are that it represents a good model of milder malaria infection and presents clear data and detailed kinetic analyses. The model offers scope for further development to better understand the consequences of altered intestinal permeability on the ongoing malaria infection, as well as predisposition to enteric pathogens.

The limitations are that the data are largely descriptive and offer limited mechanistic insight. For example, which cytokines are driving the increased intestinal permeability and what are the key cellular sources of these inflammatory mediators? Are they all systemic or are some local to the intestine? The model provides a solid basis for interrogating these and other questions.

RESPONSE:

Thank you for this very constructive and thoughtful review. Whilst this data does investigate malaria-related inflammation in a new model, we agree that more mechanistic work is now needed to identify the drivers of increased permeability and what, if any, contribution this has on promoting dissemination of secondary enteropathogens.

Competing Interests: None.