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**Article:**

Filbey, Kara J., Varyani, Fumi, Harcus, Yvonne et al. (8 more authors) (Accepted: 2019)  
Macrophage migration inhibitory factor (MIF) is essential for Type 2 effector cell immunity to an intestinal helminth parasite. *Frontiers in immunology*. ISSN 1664-3224 (In Press)

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# Macrophage migration inhibitory factor (MIF) is essential for Type 2 effector cell immunity to an intestinal helminth parasite

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**Submitted to Journal:**

Frontiers in Immunology

**Specialty Section:**

Cytokines and Soluble Mediators in Immunity

**Article type:**

Original Research Article

**Manuscript ID:**

470753

**Received on:**

07 May 2019

**Revised on:**

08 Aug 2019

**Frontiers website link:**

[www.frontiersin.org](http://www.frontiersin.org)

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### ***Conflict of interest statement***

**The 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**

### ***Author contribution statement***

KJF, FV, YH, and SL designed and undertook the majority of experiments; JPH, DJS, HMS, SN and MR undertook specialized experiments; AI provided guidance on the design of and analysed the RNA array experiment; and RMM oversaw all work and wrote the paper.

### ***Keywords***

Arginase 1 (Arg-1), helminth, macrophage, Innate immunity, eosinophil

### ***Abstract***

Word count: 255

Immunity to intestinal helminths is known to require both innate and adaptive components of the immune system activated along the Type 2 IL-4/ STAT6-dependent pathway. We have found that macrophage migration inhibitory factor (MIF) is essential for the development of effective immunity to the intestinal helminth *Heligmosomoides polygyrus*, even following vaccination which induces sterile immunity in wild-type mice. A chemical inhibitor of MIF, 4-IPP, was similarly found to compromise anti-parasite immunity. Cellular analyses found that the adaptive arm of the immune response, including IgG1 antibody responses and Th2-derived cytokines, was intact and that Foxp3+ T regulatory cell responses were unaltered in the absence of MIF. However, MIF was found to be an essential cytokine for innate cells, with ablated eosinophilia and ILC2 responses, and delayed recruitment and activation of macrophages to the M2 phenotype (expressing Arginase 1, Chil3, and RELM-alpha) upon infection of MIF-deficient mice; a macrophage deficit was also seen in wild-type BALB/c mice exposed to 4-IPP. Gene expression analysis of intestinal and lymph node tissues from MIF-deficient and -sufficient infected mice indicated significantly reduced levels of Arl2bp, encoding a factor involved in nuclear localization of STAT3. We further found that STAT3-deficient macrophages expressed less Arginase-1, and that mice lacking STAT3 in the myeloid compartment (*LysMCrexSTAT3fl/fl*) were unable to reject a secondary infection with *H. polygyrus*. We thus conclude that in the context of a Type 2 infection, MIF plays a critical role in polarizing macrophages into the protective alternatively-activated phenotype, and that STAT3 signaling may make a previously unrecognized contribution to immunity to helminths.

### ***Contribution to the field***

Our work establishes for the first time the critical role in type 2 immunity for MIF, macrophage migration inhibitory factor. As described in the manuscript, this is a long-standing member of the immunological repertoire, having been discovered in 1966. While its role in type 1 inflammation was recorded in earlier work, we now show it is a player essential for type 2 effector responses, using a helminth infection model. We use MIF-deficient mice, and confirm with a pharmacological inhibitor of MIF, that it is required for alternative activation of (M2) macrophages, and their timely expression of the key mediator Arginase-1. Gene expression analysis revealed an unexpected link with STAT3, as MIF-deficient mice also had marked reduction in a protein reported to extend nuclear localization of this factor. We therefore tested mice in which STAT3 is conditionally deleted in myeloid cells, showing a reduction in Arginase-1 responses and an inability to express functional anti-helminth immunity when challenged in a secondary infection model. We believe this manuscript will be of wide interest in its broadening of the Type 2 molecular family, both with respect to MIF itself, and also the involvement of the STAT3 pathway. The work is also timely, with the role of the canonical IL-4/ IL-13 pathway comprehensively understood, but the many additional modifiers and checkpoints of type 2 activation yet to be characterized.

### ***Funding statement***

This work was supported by the Wellcome Trust (Ref 106122) and the MRC through a CASE studentship with UCB. FV was supported through the Wellcome Trust-funded Edinburgh Clinical Academic Track, through award Ref 107490. The Wellcome Centre for Integrative Parasitology is supported by core funding from the Wellcome Trust (Ref: 104111)

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This study was carried out in accordance with the policies of the University of Glasgow and the UK Home Office. The protocols were approved by the 'University of Glasgow Ethical Review Board.

## **Data availability statement**

Generated Statement: This manuscript contains previously unpublished data. The name of the repository and accession number(s) are not available.

1 **Macrophage migration inhibitory factor (MIF) is essential for Type 2 effector cell**  
2 **immunity to an intestinal helminth parasite**

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16 **Keywords** : Arginase, *Heligmosmoides polygyrus*, helminths, macrophage, eosinophil,  
17 innate immunity

18

19 **Abstract**

20 Immunity to intestinal helminths is known to require both innate and adaptive components of the  
21 immune system activated along the Type 2 IL-4R/STAT6-dependent pathway. We have found  
22 that macrophage migration inhibitory factor (MIF) is essential for the development of effective  
23 immunity to the intestinal helminth *Heligmosomoides polygyrus*, even following vaccination  
24 which induces sterile immunity in wild-type mice. A chemical inhibitor of MIF, 4-IPP, was  
25 similarly found to compromise anti-parasite immunity. Cellular analyses found that the adaptive  
26 arm of the immune response, including IgG1 antibody responses and Th2-derived cytokines, was  
27 intact and that Foxp3<sup>+</sup> T regulatory cell responses were unaltered in the absence of MIF.  
28 However, MIF was found to be an essential cytokine for innate cells, with ablated eosinophilia  
29 and ILC2 responses, and delayed recruitment and activation of macrophages to the M2  
30 phenotype (expressing Arginase 1, Chil3, and RELM- $\alpha$ ) upon infection of MIF-deficient mice; a  
31 macrophage deficit was also seen in wild-type BALB/c mice exposed to 4-IPP. Gene expression  
32 analysis of intestinal and lymph node tissues from MIF-deficient and -sufficient infected mice  
33 indicated significantly reduced levels of *Arl2bp*, encoding a factor involved in nuclear  
34 localization of STAT3. We further found that STAT3-deficient macrophages expressed less  
35 Arginase-1, and that mice lacking STAT3 in the myeloid compartment ( $LysM^{Cre} \times STAT3^{fl/fl}$ )  
36 were unable to reject a secondary infection with *H. polygyrus*. We thus conclude that in the  
37 context of a Type 2 infection, MIF plays a critical role in polarizing macrophages into the  
38 protective alternatively-activated phenotype, and that STAT3 signaling may make a previously  
39 unrecognized contribution to immunity to helminths.

40

## 41 Introduction

42 Intestinal helminths constitute the most prevalent group of parasites in the human population  
43 today, with around 1.5 billion people infected throughout the tropical and sub-tropical zones of  
44 the globe [1; 2]. While drugs are available that temporarily clear intestinal parasites, therapy does  
45 not confer immunity to re-infection. Strategies aiming to boost the immune system through  
46 vaccination are constrained by a lack of understanding of basic mechanisms of resistance to  
47 infection, including the relative roles of innate and adaptive immunity in expelling parasites [3].  
48 Thus, while CD4<sup>+</sup> T cells are essential drivers of anti-helminth immunity, parasite expulsion  
49 requires activation of innate effector cell populations [4; 5]. In the case of the mouse model  
50 parasite *Heligmosomoides polygyrus*, the most critical effector is likely to be the alternatively  
51 activated (M2) macrophage induced through T-cell derived IL-4/-13 [6; 7]. Signaling through  
52 the IL-4R $\alpha$  subunit is essential for M2 activation, and is known to require STAT6 activation and  
53 nuclear translocation [8; 9]. However, the role of other STAT factors in immunity to helminths  
54 has been little explored [10].

55 We now identify a key player in immunity to *H. polygyrus* to be macrophage migration  
56 inhibitory factor (MIF), as mice genetically deficient in this protein, or exposed to  
57 pharmacological inhibitors of MIF, are unable to expel intestinal worms normally. Although MIF  
58 has been classically associated with type 1 inflammation during microbial exposure and sepsis  
59 [11; 12], more recent studies have also identified a role for this molecule in development of Th2  
60 responsiveness to allergens [13; 14]. It has also been reported that antibody-mediated  
61 neutralization of MIF *in vivo* increases the burden of *Schistosoma japonicum* worms in the  
62 tissues of infected mice [15]. In addition, we and others have found that M2 activation of  
63 macrophages by IL-4 is amplified in the presence of MIF [16; 17]. We therefore decided to test  
64 the role of MIF in chronic infection with *H. polygyrus*.

65 As described below, MIF-deficient animals show delayed infiltration and activation across a  
66 broad range of innate immune cell populations, including macrophages, type 2 innate lymphoid  
67 cells (ILC2s) and eosinophils. Gene array analyses of infected tissues pointed to a relatively  
68 circumscribed shift in expression profile which included sharply reduced levels of *Arl2bp*, a  
69 promoter of STAT3 function. Mice lacking STAT3 in their myeloid compartment were found to

70 phenocopy the MIF-deficient mice in failing to reject a challenge infection of *H. polygyrus*.  
71 Hence, MIF is an essential mediator in the activation of the innate compartment for  
72 immunological clearance of parasitic helminths from the gastrointestinal tract, in a manner  
73 dependent at least in part upon signaling through STAT3.

74

In review

## 75 **Materials and Methods**

### 76 **Mice and Parasites**

77 BALB/c and MIF-deficient mice on the BALB/c background [18] were bred in-house and  
78 housed in individually-ventilated cages (IVCs) according to UK Home Office guidelines. Mice  
79 on the C57BL/6 background expressing Cre recombinase under the LysM promoter [19], and  
80 carrying flanking loxP (flox) sites either side of the *Stat3* locus, were bred as described  
81 previously [20].

82 Infections employed 200 L3 larvae of *H. polygyrus* in 200 µl water by oral gavage. Parasite  
83 lifecycles and collection of HES products were conducted as previously described in  
84 CBAxC57BL/6 F1 mice [21]. Granuloma and adult worm counts were conducted after small  
85 intestines were removed and sliced longitudinally. 3-4 fecal pellets were weighed and dissolved  
86 in 2 ml dH<sub>2</sub>O; 2 ml of saturated salt solution (400 g NaCl in 1 L dH<sub>2</sub>O) was then added and eggs  
87 enumerated using a McMaster egg counting chamber. Egg counts are represented as eggs/g fecal  
88 material. For secondary infections, mice were cleared of worms after infection with *H. polygyrus*  
89 with pyrantel embonate Strongid-P paste (Elanco Animal Health), given in 2 doses of 2.5 mg  
90 dissolved in 200 µl dH<sub>2</sub>O given on days 28 and 29 by oral gavage. After 2 weeks, mice were re-  
91 infected with 200 L3 larvae.

### 92 ***In vivo* administrations**

93 1 mg of MIF inhibitor, 4-IPP (Tocris Bioscience #3249) [22] dissolved in DMSO, or DMSO  
94 alone, was administered intraperitoneally in 50 µl every other day, during *H. polygyrus* infection  
95 (adapted from [17]). 50 ng of recombinant MIF (R&D) in 50 µl PBS, or PBS alone, was  
96 administered i.p. every other day, during *H. polygyrus* infection. rIL-33 (R&D) was administered  
97 intranasally (200 ng in 50 µl PBS) to sedated mice on days 0, 1 and 2, and lung tissue taken at  
98 day 3 for analysis. *Alternaria alternata* antigen (Greer) was administered intranasally (10 µg in  
99 50 µl PBS) to sedated mice. BALF was harvested 1 hour later (adapted from [23]). For  
100 vaccination, mice were immunized with 5 µg of HES intraperitoneally in alum adjuvant, and  
101 boosted on days 28 and 35 with 1 µg in alum before challenge with *H. polygyrus* at day 42 [24].

## 102 **Cell isolation and culture**

103 MLN cell suspensions were prepared directly by passage through 70 µm nylon filters (BD) and  
104 placed in RPMI1640 (Gibco) containing 10% FCS, 1% PenStrep (Gibco) and 1% L-glutamine  
105 (Gibco)(complete RPMI). Cells were restimulated for 72 hours at 37°C with either media alone  
106 or HES at a final concentration of 1 µg/ml with 1x10<sup>6</sup> cells, in triplicate. Peritoneal exudate cells  
107 were collected by washing the peritoneal cavity with 2 x 5 ml RPMI1640 using a 23 gauge  
108 needle. Red blood cells were removed by adding 3 ml red blood cell (RBC) lysis buffer (Sigma)  
109 for 4 minutes, and washing with complete RPMI. Peritoneal lavage used for ELISA analysis  
110 consisted of the supernatant from the first 5 ml wash following centrifugation to pellet cells.  
111 Bronchoalveolar lavage was collected by washing the lungs with 1 ml ice-cold PBS. Lung tissue  
112 was digested in HBSS (Gibco) supplemented with 4U/ml Liberase TL (Roche) and 160 U/ml  
113 DNase 1 (Sigma). Tissue was incubated at 37°C for 25 mins, passed through 70 µm nylon filters  
114 (BD) and RBC-lysed before cells were used for flow cytometric analysis.

## 115 **Flow cytometry**

116 Cells were stained in 96-well round-bottomed plates. Prior to antibody staining, cells were  
117 washed in PBS and stained with LIVE/DEAD Fixable Blue (Invitrogen) at a 1/1000 dilution in  
118 100 µl PBS for 20 min at 4°C. Then, Fc receptors were blocked in 50 µl of FACS buffer  
119 containing 100 µg/ml of naïve rat IgG (Sigma) for 20 min at 4°C. Samples were then surface  
120 stained for 20 min in 20 µl of FACS buffer containing a combination of the antibodies detailed  
121 below. Lineage markers for ILC2 negative gating: CD3 (Biolegend 17A2), CD4 (Biolegend  
122 RM4-5), CD8α (Biolegend 53-6.7), CD19 (Biolegend 6D5) CD49b (eBioscience DX5), Gr1  
123 (Biolegend RB6-8C5), CD11c (Biolegend N418); ICOS (eBioscience 15F9), F4/80 (Biolegend  
124 BM8), CD11b (Biolegend M1/70), SiglecF (BD E50-2440). To measure intracellular IL-5, cells  
125 were first stimulated for 4 hrs at 37°C in the presence of PMA (50 ng/ml), Ionomycin (1 µg/ml),  
126 and Brefeldin A (10 µg/ml) (all from Sigma). Following surface staining, cells were  
127 permeabilised for 30 min at 4°C in Cytofix/Cytoperm solution (BD), and then washed twice in  
128 200 µl of Perm/Wash (BD). ILC2s were stained for intracellular cytokine expression in  
129 Perm/Wash (BD) using anti-IL-5 (eBioscience TRFK5). For Foxp3 (eBioscience, FJK-16s),  
130 Arginase-1 (R&D Systems IC5868P), RELM-α (R&D Systems 226033, labeled with AF647

131 (Invitrogen)) and Chil3 (R&D biotinylated goat anti-mouse combined with Streptavidin PeCy7  
132 (Biolegend)), samples were stained for surface markers after which cells were permeabilised for  
133 12 hrs at 4°C in Fix/Perm solution (eBioscience Foxp3 staining set), and then washed twice in  
134 200 µl of Perm/Wash (eBioscience Foxp3 staining set). After staining, cells were washed twice  
135 in 200 µl of FACS buffer before acquisition on the LSR II or Canto flow cytometers (BD  
136 Bioscience) and subsequently analysed using FlowJo (Tree Star).

### 137 **Cytokine ELISAs**

138 Cytokine levels were detected in culture supernatants and BALF by ELISA using monoclonal  
139 capture and biotinylated detection antibody pairs as follows, used at concentrations optimised  
140 previously: IL-4 (11B11 + BVD6-24G2 (BD Pharmingen)); IL-13 (eBio13A + eBio1316H  
141 (eBioscience)); IL-33 (R&D DuoSet). *p*-nitrophenyl phosphate (pNPP, 1 mg/ml, Sigma) was  
142 used as a substrate. OD was measured at 405 nm on a Precision microplate reader (Molecular  
143 Devices) and data analysed using Softmax Pro software.

### 144 **Antibody ELISAs**

145 Serum antibodies to HES were measured by ELISA as previously described [7]. Briefly, plates  
146 were coated with 1 µg/ml HES in carbonate buffer, blocked with 10% BSA in carbonate buffer,  
147 and incubated with serial dilutions of sera. Antibody binding was detected using HRP-  
148 conjugated goat anti-mouse IgA or IgG1 (Southern Biotech 1070-50 and 1040-50) and ABTS  
149 Peroxidase Substrate (KPL), and read at 405 nm.

### 150 **Gut homogenate**

151 Approx. 1 cm small intestine was homogenised in 500 µl 1x lysis buffer (Cell Signalling  
152 Technology Inc) plus 5 µl phenylmethanesulfonyl fluoride solution (PMSF) (Sigma) using a  
153 TissueLyser (Qiagen). Samples were centrifuged at 12,000 rpm for 10 mins to remove debris and  
154 supernatants added to ELISAs, at a 1:10 dilution, to measure RELM- $\alpha$  (Peprotech) and Chil3  
155 (R&D). Levels were normalised to total protein content measured using a Bradford assay. The  
156 same ELISA sets were used to analyse peritoneal lavage levels of RELM- $\alpha$  and Chi3.

## 157 **Immunohistochemistry**

158 Transverse sections were made from 2 cm of paraffin-embedded small intestine, at a thickness of  
159 4  $\mu\text{m}$  using a cryostat. For MIF staining, sections were deparaffinised by immersing slides in  
160 HistoClear (Brunel Microscopes Ltd) for 5 mins, and then hydrated through 100%, 95% and 70%  
161 ethanol successively. Antigen retrieval was undertaken with citrate buffer (20 mM citric acid +  
162 0.05% Tween 20 at pH6) warmed to 95°C for 20 mins. Sections were blocked in 1x PBS with  
163 1% BSA, 2% normal rabbit serum, 0.1% Triton X-100 and 0.05% Tween 20 for 30 mins at room  
164 temperature and then incubated with rabbit  $\alpha$ -MIF (Invitrogen) at 1:2000 dilution in block buffer,  
165 and left overnight at 4°C. Slides were immersed in 3% H<sub>2</sub>O<sub>2</sub> for 10 mins at room temperature,  
166 and washed in PBS. Goat  $\alpha$ -rabbit conjugated to biotin (Vector Laboratories) at 5  $\mu\text{g}/\text{ml}$  in PBS  
167 was added for 1 hour at room temperature, in the dark. Following 2 washes in PBS, several drops  
168 of ABC Vectastain (Vector Laboratories) were added and slides left for 30 mins at room  
169 temperature, in the dark. Slides were washed twice in PBS and DAB peroxidase solution (Vector  
170 Laboratories) was added for 5 mins (until a brown stain had developed). With water washes in  
171 between, the following were added successively to counterstain the sections: Harris hemotoxylin  
172 solution (Sigma), acid alcohol (75% ethanol + 1% HCl) and Scott's Tap Water Substitute  
173 (ddH<sub>2</sub>O + 42 mM NaHCO<sub>3</sub> and 167 mM MgSO<sub>4</sub>). Slides were dehydrated through 75%, 95%  
174 and 100% ethanol and then HistoClear added for 5 mins. Coverslips were added with DPX  
175 mountant (Sigma) and slides were left to dry overnight, in the dark. Pictures were taken using a  
176 Leica DFC290 compound microscope and Leica Application Suite software.

177 For fluorescent staining of macrophages, proximal small intestinal tissue was harvested and  
178 longitudinally opened. Any food matter was then gently removed by scraping and the tissue  
179 rolled onto a toothpick. Tissue was immediately immersed in OCT compound (Tissue-Tek),  
180 frozen on dry ice and stored at -80°C. Then, 13  $\mu\text{m}$  thick sections were cut using a cryotome  
181 (Thermo Fisher), attached to positively charged microscope slides (VWR), dried for 15 min at  
182 room temperature and then stored at -80°C. For the staining procedure, tissue sections were  
183 thawed at room temperature and dried for 15 min under airflow, followed by fixation with 4%  
184 paraformaldehyde in PBS for 15 min at room temperature. The sections were then rinsed twice in  
185 PBS, permeabilised in PBS/0.1% saponin (Sigma-Aldrich) and consecutively blocked using a  
186 solution of 10% donkey serum (Abcam) and 0.3M glycine (Fisher Scientific) in PBS for 60 min

187 at room temperature. After 2 washes in PBS/0.1% saponin, primary staining was performed  
188 overnight at 4°C in PBS/1%BSA/0.1% saponin containing an antibody cocktail of: rat anti-  
189 mouse CD68-FITC (FA-11, Biolegend used at 5ug/mL), rat anti-mouse EpCam-PE (G8.8,  
190 Biolegend, 2.5ug/mL) and sheep anti-human/mouse arginase 1 (R&D, 5ug/mL). Stained tissue  
191 sections were then washed 3 times in PBS/0.1% saponin and secondary antibody staining was  
192 performed with donkey anti-sheep AF647 (Abcam, 1/500) for 40 min at room temperature. This  
193 was followed by 3 washes in PBS/0.1% saponin and 2 PBS washes. DAPI containing  
194 Vectashield mounting media (Vector) and coverslips were applied prior to imaging using an  
195 EVOS FL Auto 2 fluorescence microscope (Invitrogen). Mean fluorescence intensity (MFI) of  
196 Arginase-1 staining in granulomas was analysed using the image analysis software Fiji (SciJava).

### 197 **RNA extraction and quantitative PCR**

198 To isolate mRNA from MLN and duodenal tissue, samples were first immersed in 1 ml of Trizol  
199 (Invitrogen) and disrupted using a TissueLyser (Qiagen) for 2 min at 25Hz and then stored at -  
200 80°C until mRNA isolation was performed with the Qiagen mRNA easy kit (Qiagen) according  
201 to manufacturer's instructions. For duodenal analysis, approx. 0.5 cm of the uppermost part of  
202 the duodenum was sampled. Briefly, tissue was first disrupted using a TissueLyser (Qiagen),  
203 then 200 µl chloroform was added and samples were centrifuged at 12,000 g for 15 min at 4°C.  
204 The upper aqueous layer was recovered and added to 500 µl of isopropanol, mixed, and stood at  
205 room temperature for 10 min. The sample was then centrifuged again at 12,000 g for 10 min at  
206 4°C. Pelleted RNA was washed once in 70% ethanol, and allowed to air dry before being  
207 dissolved in 50 µl of DEPC-treated water; 15 µl RNA was treated with DNase (DNAFree kit,  
208 Ambion), concentrations were determined using a Nanodrop 1000 (Thermo Scientific), and  
209 samples reverse-transcribed using 1-2 µg of RNA with M-MLV reverse transcriptase (Promega).  
210 A PCR block (Peltier Thermal Cycler, MJ Research) was used for the transcription reaction at  
211 37°C for 60 min. Gene transcript levels were measured by real-time PCR on a Roche Lightcycler  
212 480 II, in 10 µl total volume made up of 4 µl cDNA, 5 µl SYBR Green (Roche), 0.3 µl of each  
213 primer (10 µM), and 0.4 µl DEPC treated water (Ambion) using standard conditions for 60  
214 cycles. Target gene expression levels were normalised against the housekeeping gene GAPDH.

215 Primer sequences were as follows:

216 ARL2BP ADP-ribosylation factor-like binding protein

217 F: CGTATCCCAGGCTTCAACA

218 R: TGTGAGCAGCATGTCAAAGA

219 PHC2 Polyhomeotic 2

220 F: CCC ACA AAA TGG AAT GTA GAG G

221 R: ACT CCT CCG CGA TCT CCT:

## 222 **Array and analysis**

223 Two independent array experiments were conducted; in one, wild type BALB/c or MIF-deficient  
224 mice were infected with *H. polygyrus* for 5 days with tissues from the duodenum and MLN  
225 being collected and stored in RNA Later (Ambion) prior to processing. Duodenal tissues from  
226 uninfected BALB/c and MIF-deficient mice were also taken and stored (4 mice per group for  
227 each condition). MLNs from uninfected mice were too small to include in this experiment. In the  
228 second experiment, duodenal tissues were taken from naive mice (day 0) as well as days 3 and 7  
229 of *H. polygyrus* infection for both BALB/c and MIF-deficient mice (4 mice per group for each  
230 condition).

231 Total RNA from tissues was extracted by firstly placing tissue in RLT buffer (Invitrogen) and  
232 then homogenized using a Tissue Lyser II (Qiagen) set for 2 min at 25 Hz. RNA isolation was  
233 performed with the RNeasy mini kit (Qiagen) according to manufacturer's instructions. RNA  
234 amplification and biotinylation prior to array hybridisation was performed using the Illumina  
235 TotalPrep RNA Amplification kit (Ambion) according to manufacturer's instructions. All  
236 samples were checked for RNA quality prior to hybridization by Agilent 2100 Bioanalyzer  
237 (Agilent).

238 Data were generated at the Wellcome Trust Clinical Research Facility (WTCRF) located at  
239 Western General Hospital, Crewe Road South, Edinburgh, EH4 2XU. A total of 48 Illumina  
240 MouseWG6\_V2\_0\_R3\_11278593\_A arrays were QC analysed using the arrayQualityMetrics  
241 Bioconductor package to identify sub-standard and/or outlier arrays. Three arrays were identified  
242 as outliers and were removed from subsequent analyses.

## 243 **Software and statistics**

244 All statistical analyses were performed using Prism (Graphpad Software Inc.). Error bars on  
245 graphs display mean and standard error the mean (SEM). Student's t test was used to compare  
246 groups. n.s. = not significant, \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ . Results are combined  
247 from several similar experiments unless otherwise stated in the figure legend.

248

In review

249 **Results**

250 **Abated anti-helminth immunity in the absence of MIF**

251 A widely used model system for helminth infection is that of *H. polygyrus* in which parasitic  
252 larvae invade the intestinal tract and mature to luminal-dwelling adult worms releasing eggs into  
253 the environment [25; 26]. BALB/c mice are initially susceptible to infection but are able to  
254 gradually reduce their worm load over several weeks through a macrophage-dependent  
255 mechanism [7] , and are almost fully clear of adult worms (**Fig. 1 A**) and fecal eggs (**Fig. 1 B**)  
256 by day 28 post-infection. We tested MIF-deficient BALB/c mice and found that they were  
257 unable to reduce adult worm burdens or egg output following a primary *H. polygyrus* infection.

258 We then tested resistance of MIF<sup>-/-</sup> mice to parasite infection in two models of acquired  
259 immunity. In the first, immunity to infection can be accelerated by a prior episode of abbreviated  
260 infection, terminated by anthelmintic therapy [27]; in this case, alternatively-activated (M2)  
261 macrophages have been shown to be essential for protection [28]. We found that MIF-deficient  
262 mice are unable to expel adult worms, which are mostly cleared by day 21 in the wild-type mice  
263 (**Fig. 1 C**). Secondly, we used a vaccine model in which sterilizing immunity is elicited by  
264 immunization with *H. polygyrus* excretory-secretory (ES) products in alum adjuvant [24]. In this  
265 setting, BALB/c mice show complete protection but MIF-deficient animals fail to clear the  
266 parasites (**Fig. 1 D**).

267 We also reproduced the phenotype using a pharmacological inhibitor of MIF, 4-iodo-6-  
268 phenylpyrimidine (4-IPP), which acts as a "suicide substrate" by covalently binding the N-  
269 terminal proline required for catalytic activity [17; 22]. Mice receiving this inhibitor showed  
270 significantly greater susceptibility than vehicle-treated mice to *H. polygyrus*, in terms of both  
271 adult worm burdens and egg output (**Fig. 1 E, F**).

272 Although immunity generally correlates with the formation of intestinal granulomas [29; 30], we  
273 found that MIF<sup>-/-</sup> mice developed normal numbers of granulomas despite being completely  
274 susceptible to infection (**Fig. 1 G**).

## 275 **Intact adaptive Type 2 responses in MIF-deficient mice**

276 Immunity to *H. polygyrus* following either drug-mediated clearance, or HES vaccination, has  
277 been shown to be antibody-dependent, in particular requiring IgG1 [24; 31]. We therefore  
278 compared serological responses to infection in wild-type and MIF<sup>-/-</sup> mice but found no  
279 difference in serum IgG1 titer (**Fig. 2 A**). In addition, both genotypes responded with equally  
280 high anti-HES antibody titers following vaccination (**Fig. 2 B**), although only the MIF<sup>-/-</sup> animals  
281 failed to expel parasites. These findings implicated a deficiency in the cellular arm of the  
282 response in the absence of MIF.

283 We then compared parasite-specific T cell responses, as immunity to *H. polygyrus* is strongly  
284 Th2 dependent [32], by challenging small intestine draining mesenteric lymph node (MLN) cells  
285 from *H. polygyrus*-infected BALB/c and MIF<sup>-/-</sup> mice with HES antigens. We found comparably  
286 robust IL-4 and IL-13 responses in both strains (**Fig. 2 C, D**); no induction of antigen-specific  
287 IFN $\gamma$  responses above background was seen in either strain (data not shown), indicating the  
288 susceptibility of the MIF-deficient mouse cannot be explained by a switch to the Th1 mode of  
289 immunity.

290 Regulatory T cells (Treg) expressing the Foxp3<sup>+</sup> transcription factor are known to expand during  
291 *H. polygyrus* infection [33; 34] and render mice susceptible [35; 36]. MLN cell populations were  
292 analyzed by flow cytometry at 14 and 28 days post-infection, and similar increases in Foxp3<sup>+</sup>  
293 Treg numbers were seen in both wild-type and MIF<sup>-/-</sup> mice infected with *H. polygyrus* (**Fig. 2 E**).  
294 Increases in Treg frequency (as percentage of total CD4<sup>+</sup> cells) and Foxp3 intensity were also  
295 similar between the two strains (data not shown), indicating that increased Treg activity is not  
296 contributing to greater susceptibility in the gene-targeted mice.

## 297 **Impaired innate Type 2 responses in MIF-deficient mice**

298 We then analyzed innate immune cell responses in BALB/c and MIF<sup>-/-</sup> mice at day 7 post-*H.*  
299 *polygyrus* infection. In the wild-type animals, infection provokes a sharp increase in cell  
300 numbers within the MLNs (**Fig 3 A**), which is diminished in the MIF<sup>-/-</sup> mice. Infection also  
301 results in activation of ILC2s to express IL-5 which is almost totally ablated in the MIF-deficient  
302 animals (**Fig. 3 B**). Notably, while the proportion of ILCs within the lymph nodes of both  
303 genotypes are similar, fewer are IL-5<sup>+</sup> (**Fig 3 C**). Likewise, cellular expansion in the peritoneal

304 cavity provoked by infection is profoundly reduced in MIF-deficient mice (**Fig. 3 D**), as is  
305 eosinophilia (**Fig. 3 E**). The loss of eosinophils in the absence of MIF has previously been  
306 observed in both helminth infection and airway asthma models [13; 37; 38]. We also tested the  
307 MIF-dependence of eosinophilia by administering the 4-IPP MIF inhibitor at the time of *H.*  
308 *polygyrus* infection. Wild-type mice receiving this inhibitor showed significantly reduced  
309 peritoneal eosinophilia compared to animals treated with the DMSO vehicle alone (**Fig. 3 F**).

310 To ascertain whether ILC2 differentiation was intrinsically compromised in MIF-deficient mice,  
311 we first tested the effects of exogenous IL-33 injection on the activation of ILCs in the lung; IL-  
312 33 drove equivalent IL-5<sup>+</sup> ILC2 responses irrespective of MIF genotype (**Fig. 3 G**). We then  
313 tested the response of mice to airway challenge with *Alternaria* allergen, a potent stimulator of  
314 the ILC2 population through provoking rapid release of IL-33 from the airway epithelium [39].  
315 The introduction of exogenous *Alternaria* antigen elicited equivalent levels of IL-33 into the  
316 bronchoalveolar lavage after 1 hr in BALB/c and MIF-deficient animals (**Fig. 3 H**), arguing that  
317 both the release of ILC2-stimulating alarmins and the development of ILC2 responses to these  
318 cytokines are intact in the MIF-deficient setting.

319 The diminished eosinophil responses in MIF-deficient mice cannot readily account for their  
320 increased susceptibility, as eosinophil-deficient  $\Delta$ dblGATA mice retain their ability to expel *H.*  
321 *polygyrus* following vaccination [24]. In addition, the poor ILC2 response observed did not  
322 translate into any shortfall in the Th2 response that develops to parasite antigens (**Fig. 2 C, D**),  
323 although it is possible that abated ILC2 production of IL-5 explains the deficient eosinophil  
324 responses in MIF<sup>-/-</sup> mice.

## 325 **Type 2 myeloid responses in MIF-deficient mice**

326 We next analyzed myeloid subpopulations, which play critical roles in mediating immunity in  
327 many helminth settings [7; 28; 40]. To establish whether the absence of MIF resulted in  
328 significant differences within the myeloid compartment, we compared the phenotype of CD11b<sup>+</sup>  
329 F4/80<sup>+</sup> macrophages in MIF-sufficient and -deficient mice. We found that, following *H.*  
330 *polygyrus* infection, few viable lamina propria cells could be recovered from either BALB/c or  
331 MIF-deficient mice and hence populations were assayed from the peritoneal cavity, in which  
332 there is extensive expansion and alternative activation of macrophages during the first week of

333 infection [41]. Notably, the increase in macrophage numbers was muted in the peritoneal cavity  
334 of MIF-deficient mice (27% above naïve levels) compared to wild-type animals (90% increased)  
335 (**Fig. 4 A**).

336 Because MIF has previously been shown to promote the alternative activation of macrophages,  
337 alongside IL-4R $\alpha$ -binding cytokines [16], we measured expression of key alternatively-activated  
338 macrophage (AAM)-associated gene products Arginase-1, Chil3 (Ym1) and RELM $\alpha$  by a  
339 combination of flow cytometry of peritoneal cell populations, and ELISA for soluble proteins in  
340 peritoneal lavage fluids. By each of these measures MIF<sup>-/-</sup> mice showed significant impairment  
341 of alternative activation. Thus, the proportions of peritoneal macrophages staining for Arginase-1  
342 (**Fig. 4 B, C**) and RELM $\alpha$  (**Fig. 4 D, E**) were significantly reduced in MIF-deficient animals, as  
343 were levels of detectable Chil3 and RELM $\alpha$  protein in the lavage following *H. polygyrus*  
344 infection (see below).

345 We next examined the *in vivo* effects of pharmacological MIF inhibition on the expression of  
346 AAM markers; administration of 4-IPP significantly reduced the number of CD11b<sup>+</sup> Arginase-1<sup>+</sup>  
347 (**Fig. 4 F**) and Chil3<sup>+</sup> (**Fig. 4 G**) peritoneal macrophages after *H. polygyrus* infection, as well as  
348 the levels of both Chil3 and RELM $\alpha$  protein in the peritoneal lavage fluid of BALB/c mice (**Fig.**  
349 **4 H**).

350 To test whether MIF is directly responsible for the alternative activation of macrophages, we  
351 evaluated the effects of administering recombinant MIF into the peritoneal cavity of MIF-  
352 deficient mice. Such treatment restored the proportions of Chil3<sup>+</sup> AAM in this site after *H.*  
353 *polygyrus* infection to levels comparable with wild-type mice (**Fig. 5 A**), but did not rescue the  
354 significant deficit in ILC2 cells in the same location (**Fig. 5 B**). Exogenous MIF was able to  
355 partially restore protein levels of Chil3 and RELM- $\alpha$  in the peritoneal lavage fluid of MIF<sup>-/-</sup> mice  
356 (**Fig. 5 C, D**), although remaining significantly below those of the wild-type mice, and no  
357 eosinophilia was elicited (data not shown). Furthermore, these products were also upregulated in  
358 small intestinal tissues of *H. polygyrus*-infected MIF-deficient mice (**Fig. 5 E, F**). As  
359 intraperitoneal delivery of MIF did not restore resistance to the parasite infection (data not  
360 shown), it is likely that localized production and release within the intestinal tract may be  
361 required for effective recruitment and activation of tissue macrophages at the site of infection.

362 While peritoneal macrophages may mirror the phenotype of the intestinal population, it is  
363 important to also study those cells closely associated with larval parasites in the submucosa of  
364 the small intestine, where *H. polygyrus* is found for the first 8 days of infection. We used  
365 immunofluorescence imaging to characterise the patterns of macrophage activation and  
366 accumulation around larval parasites, and their expression of Arginase-1 which is known to be  
367 required for immunity to this helminth [28]. Surprisingly, local macrophage infiltration and  
368 overall Arginase-1 expression did not significantly differ in infected MIF<sup>-/-</sup> mice (**Fig. 6 A**), and  
369 although the intensity of Arginase-1 staining in gene-deficient tissues was marginally weaker at  
370 day 4 of infection (**Fig. 6 B**), by day 6 it was as ubiquitous as in the wild-type controls (**Fig. 6**  
371 **C**). In both examples, Arginase-1 is disseminated throughout the granuloma, indicating that it is  
372 either or both expressed by cells other than macrophages, and/or released extensively into the  
373 extracellular milieu from those cells which express it. Immunohistochemical staining was also  
374 used to identify widespread expression of MIF in intestinal tissues *in vivo*; in particular, MIF was  
375 intensely expressed within the granulomas centered around immobile larvae (**Fig. 6 D**), at the  
376 foci of the local immune response to intestinal helminth infection.

#### 377 *Gene expression in H. polygyrus-infected MIF-deficient mice*

378 To gain insight into possible signaling and effector molecules dependent upon MIF in helminth  
379 infection, we compared gene expression profiles of MIF-sufficient and -deficient mice by array  
380 analyses of duodenal tissue taken 3, 5 and 7 days following *H. polygyrus* infection, as well as  
381 MLN sampled on day 5. As shown in **Fig. 7 A, B**, relatively few genes showed major expression  
382 changes but among them were *Arl2bp*, a little-studied gene encoding a protein which stabilizes  
383 nuclear localization of the STAT3 transcription factor [42], and *Phc2*, a central component of the  
384 Polycomb 1 complex that maintains epigenetic imprinting [43]. In addition, a number of other  
385 genes showed either smaller or more transient reductions in levels in *H. polygyrus*-infected MIF<sup>-</sup>  
386 <sup>-</sup> mice compared to wild-type, including *Retnlb* (encoding RELM-β) and *Pla2g1b*, two  
387 epithelial-expressed genes reported to possess direct anti-helminth properties [44; 45].

#### 388 *STAT3 signaling contributes to immunity to H. polygyrus*

389 Gene expression differences for *Arl2bp* and *Phc2* were confirmed by RT-PCR on MLN samples  
390 at day 5 of infection of wild-type and MIF-deficient mice (**Fig. 7 C, D**). As abated *Arl2bp*

391 expression may compromise STAT3 signaling, we then tested mice in which STAT3 expression  
392 had been blocked in myeloid lineages through transgenic expression of Cre recombinase under  
393 the LysM promoter, combined with homozygous alleles for a flox-flanked STAT3 [46]. We first  
394 examined CD11b<sup>+</sup>F4/80<sup>+</sup> peritoneal macrophages from *H. polygyrus*-infected mice, and found  
395 significantly fewer cells expressed intracellular Arginase-1 in mice carrying the myeloid-  
396 restricted deletion (**Fig. 8 A**); notably, Chil3<sup>+</sup> cell numbers were similar, albeit low, in both  
397 genotypes (**Fig. 8 B**). We also collected peritoneal lavage fluid, and found significantly lower  
398 Arginase enzymatic activity in the STAT3-conditionally deleted mice (**Fig. 8 C**). In addition, we  
399 measured soluble Chil3 in the lavage fluid, which rather than being inhibited in mice lacking  
400 myeloid cell STAT3 expression, actually showed a significant increase (**Fig. 8 D**).

401 As the C57BL/6 background is fully susceptible to primary *H. polygyrus* infection, we then  
402 evaluated immunity to a secondary challenge with *H. polygyrus* following chemotherapeutic  
403 clearance of the primary infection. In wild-type mice lacking the Cre allele, there was significant  
404 protection against challenge, but in myeloid-specific STAT3-deleted animals, parasite loads were  
405 similar in primary and secondary infection, showing a failure of protective immunity in the  
406 STAT3-deficient setting (**Fig.8 E**).

407

408 **Discussion**

409 The crucial role of innate immune cell populations in immunity to helminths is well recognized  
410 [5; 47], but the molecular mediators required for their activation have not all been identified.  
411 Here we report that MIF is a critical cytokine required for clearance of the intestinal parasite *H.*  
412 *polygyrus*, impacting on multiple type 2 innate cell populations while not significantly affecting  
413 adaptive B or T cell responses. Although previously viewed as a pro-inflammatory agent in  
414 settings of sepsis and microbial challenge [48], our work and that of others demonstrate that its  
415 role is context-dependent, so that in the presence of the pivotal type 2 cytokine IL-4, MIF will  
416 synergise to induce characteristic M2 products including Chil3, RELM $\alpha$  and Arginase-1 [16;  
417 17]. Significantly, the activity of MIF is not confined to the macrophage lineage, with evident  
418 lesions in ILC numbers, and a profound loss of eosinophils, in MIF-deficient animals. These  
419 multiple facets of MIF are characteristic of a protein with a range of diverse activities that are  
420 remarkable for a protein of only 114 amino acids, and one discovered at the dawn of the cytokine  
421 era [49; 50]. For example, MIF is also a nonconsensus ligand of chemokine receptors [51], an  
422 inhibitor of intracellular signaling and inflammasome assembly [52; 53] and a partner in a  
423 nuclear DNA-cleaving complex [54].

424 Immunity to *H. polygyrus* is known to require a potent type 2 response, dependent upon CD4<sup>+</sup>  
425 Th2 cells driving a specific IgG1 antibody response together with alternatively-activated (M2)  
426 macrophages stimulated through the IL-4R [7; 28; 55]. Immunity can act in two distinct phases:  
427 firstly against tissue-dwelling larvae which are immobilised and killed in the setting of a  
428 challenge infection or an immunized host, and secondly against luminal adults which are cleared  
429 by the combined action of activated myeloid and epithelial cells [26]. Importantly, parasites  
430 surviving immune attack in the tissues can emerge into the lumen with diminished fitness,  
431 resulting in lower egg production and shorter survival times. MIF deficiency was found not only  
432 to compromise worm expulsion in both naive and vaccinated animals, but also to result in  
433 significantly higher egg production at day 14 (**Fig 1 B**), confirming that early responses to the  
434 tissue larvae are abated in the absence of MIF.

435 Among other innate cell populations, ILC2s can promote the response, but are not sufficient for  
436 expulsion [56], while eosinophils act to restrain the intestinal Th2 response to *H. polygyrus* [57]

437 and eosinophil-deficient mice clear parasites promptly following immunization with a secreted  
438 antigen vaccine [24]. As MIF-deficient mice mounted a normal adaptive B- and T cell response  
439 to infection, we concluded that these mice must lack a key innate effector population, which we  
440 propose are the IL-4R-dependent M2 macrophages. Indeed, even in the presence of memory Th2  
441 cells known to drive M2-dependent immunity to *H. polygyrus* [28] immunity fails in the absence  
442 of MIF, again suggesting lesion(s) in the macrophage compartment. If so, this would argue that  
443 IL-4R ligation may require supplementation through other signals to achieve the fully activated  
444 M2 state required for worm expulsion. A further interesting point is that MIF-deficient mice  
445 generated a similar granuloma response to wild-type animals, and yet could not trap parasites  
446 even following vaccination. As cell recruitment to granulomas was similar in the two genotypes,  
447 and as MIF itself is highly expressed in the WT granuloma, it may be that MIF acts not at the  
448 level of differential cell recruitment, but by activating cells locally to promote immunity.

449 It is known that numbers of macrophages in *H. polygyrus* infection rapidly expand, notably in  
450 the peritoneal cavity, and adopt the M2 phenotype characterized by production of Arginase-1,  
451 Chil3 and RELM $\alpha$  [41]. We noted a significant delay in peritoneal macrophage activation in  
452 MIF-deficient animals, with a lag also evident in production of these archetypal markers,  
453 although the effect was less obvious at the tissue site of infection. However, it is known that  
454 genetically resistant strains of mice mount a more rapid response to *H. polygyrus* [7], suggesting  
455 that retardation of M2 activation in the absence of MIF may account for failure of immunity.  
456 We further established the role of MIF in testing a pharmacological inhibitor, which  
457 recapitulated both impaired expression of M2 gene products, and greater susceptibility to  
458 infection, that are observed in the gene-targeted mice. In addition, we established exogenous  
459 MIF could restore the activation of peritoneal macrophages to the wild-type profile.

460 Previous work has demonstrated that immunity to *H. polygyrus* is compromised by clodronate  
461 depletion of macrophages [7], and by pharmacological blockade of arginase-1, a principal  
462 product of M2 macrophages [28]. However, we have yet to establish whether such macrophages  
463 activated by MIF would be sufficient to confer immunity by adoptive transfer to naive recipients.  
464 One obstacle to such an experiment is that appropriate migration of the adoptively transferred  
465 population to the site of infection may not occur, particularly given that MIF is thought to arrest  
466 macrophage migration in situ. We note, however, that in a related parasite model of

467 *Nippostrongylus brasiliensis*, in which larvae transit the lung intranasal transfer of M2  
468 macrophages significantly augments anti-parasite immunity [58].

469 To further analyse the role of MIF *in vivo*, we next compared gene expression profiles, in the  
470 intestinal tract and MLN. In these tissues, disparities in M2 macrophage products were not so  
471 apparent, but two transcripts markedly under-represented in the MIF-deficient state were *Arl2bp*  
472 and *Phc2*. The former is a STAT3 nuclear retention factor, raising the possibility that STAT3 is  
473 required for functional macrophage activity in *H. polygyrus* infection. While we did not observe  
474 any difference in STAT3 phosphorylation following IL-6 or IL-10 stimulation of wild-type or  
475 MIF-deficient cells (data not shown), *Arl2bp* may manifest a more subtle effect on nuclear  
476 activity which we could not detect. However, it has previously been shown that Arginase-1 and  
477 *Chil3* expression are STAT-3 dependent in mammary epithelial cells [59], as is also the case for  
478 Arginase-1 in myeloid-derived suppressor cells (MDSCs) [60], which share some characteristics  
479 with M2 macrophages. Although global STAT3 deletion is lethal in mice, we were able to test  
480 animals with a myeloid-specific conditional deletion of STAT3, which show modest reduction in  
481 Arginase-1 expression during *H. polygyrus* infection and lose the ability to expel parasites on  
482 secondary exposure. As pharmacological inhibition of arginase is also able to block expulsion of  
483 this parasite [28], these data may indicate that small changes in the timing or peak of arginase  
484 production are sufficient to alter the outcome of infection. In addition, our finding that *Chil3* is  
485 actually increased in myeloid-specific STAT3-deficient mice which fail to expel, would argue  
486 that despite its abundance, *Chil3* is not a primary factor that promotes helminth clearance.

487 As mentioned above, MDSCs commonly express arginase. They are also expanded *in vitro* by  
488 MIF [17; 61; 62], and their development is in part STAT3-dependent [63]. Furthermore, MDSC  
489 transfer alone has been reported to hasten expulsion of the nematode species *N. brasiliensis* from  
490 mice [64]. In addition, key enzymes such as amylases (*Amy2a5*, **Fig. 7 B**) which show ablated  
491 gene expression in MIF-deficient mice are reported to be up-regulated in tumor-associated  
492 MDSCs [65]. However, MDSCs are a highly heterogenous grouping of myeloid cells [66], and  
493 further definition of which, if any, subset of these cells may play a role in helminth immunity  
494 will be an important future goal.

495 While our data argue that MIF, and STAT3, are each involved in macrophage immune function,  
496 we cannot exclude that other cell types respond to these signals, and are integrally required for  
497 parasite expulsion. For example, neutrophils play a role particularly in primary infection, as  
498 depletion of Gr1<sup>+</sup> cells compromises primary immunity to *H. polygyrus* [24; 67] and can prime  
499 macrophages for resistance to challenge infection with this parasite [68].

500 Finally, in mice lacking MIF there was a substantial reduction of a polycomb 1 complex gene,  
501 *Phc2*, which we are now exploring. The polycomb complex mediates chromosomal imprinting  
502 [43] which is a central feature of macrophage commitment and innate immune memory [69; 70;  
503 71]. While *Phc2* itself has yet to be implicated in macrophage differentiation, other polycomb  
504 components are known to be involved [72], and epigenetic modifications have been found to be  
505 essential to the phenotypes of both M1 [73; 74] and M2 [75; 76] macrophages. Hence, there may  
506 be a longer-term inability of macrophages to fully polarize and form innate memory in the  
507 absence of MIF, which in turn could explain the failure to expel parasites in vaccinated animals.

508 With this report, MIF may be seen as joining the ranks of intestinal epithelial-derived mediators  
509 that recruit and sustain innate immune responses. However, MIF is produced and functions in  
510 many niches, and many critical features underpinning the source, stimulus and regulation of its  
511 expression remain to be determined. The discovery that additional stimuli are required for  
512 optimal alternative activation will also be important in defining the pathway through which the  
513 M2 phenotype is controlled. Indeed, a number of co-activating pathways for M2 macrophages  
514 have very recently been described including surfactant protein A [77] and markers of apoptosis  
515 [78] – as with MIF these ligands may prove indispensable in designing future interventions to  
516 generate protective immunity to the range of parasitic organisms for which type 2 immunity is  
517 critical.

518

519 **References**

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788 **Acknowledgments**

789 We thank Nicola Britton for excellent technical assistance.

790 **Funding**

791 This work was supported by the Wellcome Trust (Ref 106122) and the MRC through a CASE  
792 studentship with UCB. FV was supported through the Wellcome Trust-funded Edinburgh  
793 Clinical Academic Track, through award Ref 107490. The Wellcome Centre for Integrative  
794 Parasitology is supported by core funding from the Wellcome Trust (Ref: 104111)

795 **Author contributions**

796 KJF designed and undertook the majority of experiments; FV, JPH, DJS, SN and SL undertook  
797 experiments; AI provided guidance on the design of and analysed the RNA array experiment; SN  
798 & MR undertook the STAT3 flox experiments; MR and HJM provided guidance and expertise,  
799 SL critically reviewed the manuscript, RMM oversaw all work and wrote the paper.

800 **Competing interests**

801 The authors declare no competing financial interests.

802

803 **FIGURES AND FIGURE LEGENDS**

804 **Figure 1 - Compromised anti-helminth immunity in the absence of MIF**

805 **A, B.** Differential susceptibility of BALB/c and MIF<sup>-/-</sup> mice to primary infection with *H. polygyrus*.

806 Adult worm burdens in the small intestine (**A**) and fecal egg counts (**B**) were determined at  
807 days 14 and 28 post-infection with 200 *H. polygyrus* larvae by gavage. Data shown are  
808 combined from 3 independent experiments.

809 **C.** Differential induction of immunity following drug-abbreviated primary infection in BALB/c  
810 and MIF<sup>-/-</sup> mice. At day 28 following infection with *H. polygyrus*, mice were given 2.5 mg of  
811 pyrantel embonate by oral gavage, twice over 24 hours. After a further 14 days, mice were  
812 reinfected (or infected for the first time in 1° groups). Adult worms were enumerated at day 21  
813 post infection. Data shown are combined from 2 independent experiments.

814 **D.** Differential expression of vaccine-induced immunity in BALB/c and MIF<sup>-/-</sup> mice. At day 0,  
815 mice were injected with 10 µg of HES in alum, or PBS-alum control, followed by booster  
816 injections of 1 µg HES or PBS at days 28 and 35. On day 42 all mice were infected with *H.*  
817 *polygyrus*. Adult worm burdens were counted at day 21 post-infection. Data shown are  
818 combined from 2 independent experiments.

819 **E, F.** The MIF inhibitor 4-iodo-6-phenylpyrimidine (4-IPP) inhibits immunity in BALB/c mice  
820 infected with *H. polygyrus*. 1 mg 4-IPP in DMSO or DMSO alone was injected i.p. at days -1,  
821 0, 2, 4 and 6 post-infection. Adult worms (**F**) and egg burdens (**G**) were enumerated at day 28  
822 post-infection. Data shown are combined from 2 independent experiments.

823 **G.** Numbers of intestinal granulomas in BALB/c and MIF<sup>-/-</sup> mice 14 days following primary  
824 infection with *H. polygyrus*. Data shown are combined from 2 independent experiments.

825 n.s. = not significant, \* = p<0.05, \*\*\* = p<0.001, \*\*\*\* = p<0.0001

826

827 **Figure 2 - Intact adaptive and regulatory responses in MIF-deficient mice**

828 **A.** Comparable anti-helminth humoral immunity in BALB/c and MIF<sup>-/-</sup> mice. Titers of HES-  
829 specific IgG1 serum antibodies from naïve and day 28-infected BALB/c and MIF<sup>-/-</sup> mice,  
830 assessed by ELISA. Data are representative of two independent experiments.

831 **B.** Comparable anti-helminth humoral immunity in HES-vaccinated BALB/c and MIF<sup>-/-</sup> mice.  
832 Parasite-specific antibody responses in vaccinated BALB/c and MIF<sup>-/-</sup> mice. HES-specific  
833 serum IgG1 levels were measured by ELISA on the day of challenge infection. Data are  
834 representative of two independent experiments.

835 **C, D.** Comparable adaptive type 2 immune responses in BALB/c and MIF<sup>-/-</sup> mice. Th2 cytokines  
836 from culture medium of MLNC from naïve and day 14 *H. polygyrus*-infected BALB/c and  
837 MIF<sup>-/-</sup> mice, restimulated with 1 µg/ml HES or media for 72 hours. Levels of IL-4 (**C**) and IL-  
838 13 (**D**) were measured by ELISA. Data are representative of two independent experiments.

839 **E.** Regulatory cell induction by helminth infection is comparable between BALB/c and MIF<sup>-/-</sup>  
840 mice. Numbers of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells within MLNs from BALB/c and MIF<sup>-/-</sup> mice at  
841 days 14 and 28 post-infection with *H. polygyrus*. Data are representative of two independent  
842 experiments.

843 n.s. = not significant, \*\* = p<0.01, \*\*\* = p<0.001

844

845 **Figure 3 - Impaired innate type 2 responses in MIF-deficient mice**

- 846 **A.** Total numbers of MLN cells recovered from BALB/c and MIF<sup>-/-</sup> mice 7 days following *H.*  
847 *polygyrus* infection. Data are representative of two independent experiments.
- 848 **B.** Differential induction of ILC2s in BALB/c and MIF<sup>-/-</sup> mice following *H. polygyrus* infection.  
849 Total numbers of IL-5<sup>+</sup> ICOS<sup>+</sup> lineage<sup>-</sup> ILC2s within MLNC from naïve and d7 *H. polygyrus*-  
850 infected BALB/c and MIF<sup>-/-</sup> mice. Data are representative of two independent experiments.
- 851 **C.** Representative flow cytometry plots of ICOS vs Lineage markers within CD4<sup>-</sup> MLN cells from  
852 naïve BALB/c and MIF<sup>-/-</sup> mice or 7 days following *H. polygyrus* infection.
- 853 **D.** Total numbers of peritoneal lavage cells recovered from BALB/c and MIF<sup>-/-</sup> mice 7 days  
854 following *H. polygyrus* infection. Data are representative of two independent experiments.
- 855 **E.** Differential induction of eosinophilia in BALB/c and MIF<sup>-/-</sup> mice following *H. polygyrus*  
856 infection. Total numbers of eosinophils (SiglecF<sup>+</sup> CD11b<sup>+</sup>) in naïve and *H. polygyrus*-infected  
857 BALB/c or MIF<sup>-/-</sup> mice within the peritoneal lavage, at day 7 post-infection. Data are  
858 representative of two independent experiments.
- 859 **F.** The MIF inhibitor 4-IPP inhibits eosinophilia in BALB/c mice infected with *H. polygyrus*.  
860 Eosinophil numbers at day 7 post-infection with *H. polygyrus* in BALB/c following  
861 administration of 1 mg of the MIF inhibitor, 4-IPP, assessed as SiglecF<sup>+</sup>CD11b<sup>+</sup> cells within  
862 the peritoneal lavage. Results are combined from two experiments with similar results.
- 863 **G.** MIF<sup>-/-</sup> mice do not have an intrinsic defect in ILC2 induction. IL-5<sup>+</sup> ILC2s as a proportion of  
864 live cells in digested lung tissue of BALB/c or MIF<sup>-/-</sup> mice treated intranasally with PBS or rIL-  
865 33, measured by flow cytometry. Data are representative of two independent experiments, and  
866 were analyzed by nonparametric statistics.
- 867 **H.** MIF<sup>-/-</sup> mice have normal ability to release the key alarmin IL-33 upon stimulation. Levels of  
868 IL-33 in BALF of BALB/c or MIF<sup>-/-</sup> mice 1 hour after intranasal administration of 10 µg  
869 *Alternaria* antigen, measured by ELISA. Results are combined from two experiments with  
870 similar results.

871 n.s. = not significant, \* = p < 0.05, \*\* = p < 0.01, \*\*\* = p < 0.001

872

873 **Figure 4 - Type 2 myeloid responses in MIF-deficient mice**

- 874 **A.** MIF<sup>-/-</sup> mice have a reduced capacity for induction of macrophages after helminth infection.  
875 Total macrophage (CD11b<sup>+</sup>F4/80<sup>+</sup>) numbers within the peritoneal cavity of BALB/c and MIF<sup>-/-</sup>  
876 mice at day 6 post-infection with *H. polygyrus*, or in naïve mice. Data are representative of  
877 two independent experiments.
- 878 **B.** Percentage of peritoneal macrophages that are Arginase<sup>+</sup> in BALB/c and MIF<sup>-/-</sup> mice at day 3  
879 post-infection with *H. polygyrus*, or in naïve mice. Results are combined from two experiments  
880 with similar results.
- 881 **C.** Representative flow cytometry plots of CD11b and Arginase-1 staining in peritoneal  
882 macrophages from BALB/c and MIF<sup>-/-</sup> mice.
- 883 **D.** Percentage of peritoneal macrophages that are RELMα<sup>+</sup> in BALB/c and MIF<sup>-/-</sup> mice at day 6  
884 post-infection with *H. polygyrus*, or in naïve mice (D) and . Data are representative of two  
885 independent experiments.
- 886 **E** Representative flow cytometry plots of CD11b and RELM-α staining in peritoneal  
887 macrophages from BALB/c and MIF<sup>-/-</sup> mice.
- 888 **F.** The MIF inhibitor, 4IPP, can replicate the macrophage deficit of MIF<sup>-/-</sup> mice in BALB/c mice  
889 after *H. polygyrus* infection. Percentage of peritoneal macrophages that are Arginase-1<sup>+</sup> in  
890 DMSO- or 4IPP- treated BALB/c mice at day 3 post-infection with *H. polygyrus*, or in naïve  
891 mice receiving no treatment. Data are representative of two independent experiments.
- 892 **G.** Number of peritoneal macrophages that are Chil3<sup>+</sup> in DMSO- or 4IPP-treated BALB/c mice at  
893 day 7 post-infection with *H. polygyrus*. Results are combined from two experiments with  
894 similar results.
- 895 **H.** Levels of RELMα and Chil3 measured by ELISA in peritoneal lavage fluid of DMSO- or  
896 4IPP- treated BALB/c mice at day 7 post-infection with *H. polygyrus*. Data are representative  
897 of two independent experiments.

898 \* = p < 0.05, \*\* = p < 0.01, \*\*\* = p < 0.001

899

900 **Figure 5 – rMIF rescues the macrophage phenotype of MIF-deficient mice**

901 **A.** Administration of recombinant MIF can rescue the MIF<sup>-/-</sup> phenotype after *H. polygyrus*  
902 infection. Percentage of peritoneal macrophages (CD11b<sup>+</sup> F4/80<sup>+</sup>) that are Chil3<sup>+</sup> in BALB/c  
903 or MIF<sup>-/-</sup> mice treated with PBS or 50 µg recombinant MIF intraperitoneally, at day 7 post-  
904 infection with *H. polygyrus*, or in naïve mice. Results are combined from two experiments  
905 with similar results.

906 **B.** Percentage of PL cells that are IL-5<sup>+</sup> ILC2s from the same experiments as (A). Results are  
907 combined from two experiments with similar results.

908 **C, D.** Expression of Chil3 (C) and RELMα (D) in peritoneal lavage fluid, measured by ELISA, from  
909 the same experiments as (A). Results are combined from two experiments with similar results.

910 **E, F.** Expression of Chil3 (E) and RELMα (F) in small intestinal homogenate, measured by ELISA,  
911 from the same experiments as (A). Results are combined from two experiments with similar  
912 results.

913 n.s. = not significant, \* = p<0.05, \*\* = p<0.01, \*\*\* p=<0.001.

914

915 **Figure 6 - Myeloid cell infiltration around tissue larvae of *H. polygyrus* with expression of**  
916 **Arginase-1 and MIF**

917 **A.** M2 macrophages infiltrate the infection site of *H. polygyrus* larvae in the small intestine.  
918 Arginase, CD68 and EpCam staining of granulomas around larval parasites in the small  
919 intestinal submucosa at days 4 and 6 of *H. polygyrus* infection in BALB/c and MIF<sup>-/-</sup> mice.  
920 Images are representative of two experiments with similar results.

921 **B, C.** Analysis of intensity of Arginase staining in granulomas at days 4 and 6 after *H. polygyrus*  
922 infection. For each group at each time point, granulomas were analyzed from 4 individual  
923 animals, and intensity data pooled. Each data point represents an individual granuloma.

924 **D.** MIF is expressed in the infection site of *H. polygyrus* larvae in the small intestine. Intense  
925 staining of polyclonal rabbit anti-MIF antibody is observed in the granulomas around larval  
926 parasites in the small intestinal submucosa at day 6 of *H. polygyrus* infection in BALB/c mice,  
927 but not in MIF<sup>-/-</sup> animals or in sections stained with isotype control IgG. In addition,  
928 widespread specific antibody staining is seen throughout the submucosal tissue. Images were  
929 collected on a Leica compound microscope. Scale bars represent 500 and 250  $\mu\text{m}$ .

930 **Figure 7 - Gene expression in *H. polygyrus*-infected MIF-deficient mice**

931 **A.** Volcano plot comparing gene expression in duodenal tissue from BALB/c and MIF<sup>-/-</sup> mice 5  
932 days after *H. polygyrus* infection. Genes of interest are labeled in red; other loci showing large  
933 and/or significant changes are labeled in blue.

934 **B.** Heat maps from two gene expression experiments, on duodenal and MLN tissue for expression  
935 of selected genes 5 days after *H. polygyrus* infection in BALB/c and MIF<sup>-/-</sup> mice (left hand  
936 panel), and of duodenal tissues at days 3 and 5 post-infection in the same genotype mice (right  
937 hand panel). Expression levels are colored from blue (lowest) to yellow(highest) and in each  
938 case represent the mean of 4 replicates.

939 **C, D.** RT-PCR validation of *Arl2bp* (**C**) and *Phc2* (**D**). MLNs were harvested for analysis at d 5 of *H.*  
940 *polygyrus* infection, and subject to RT-PCR using gene specific primers.

941 hima

942

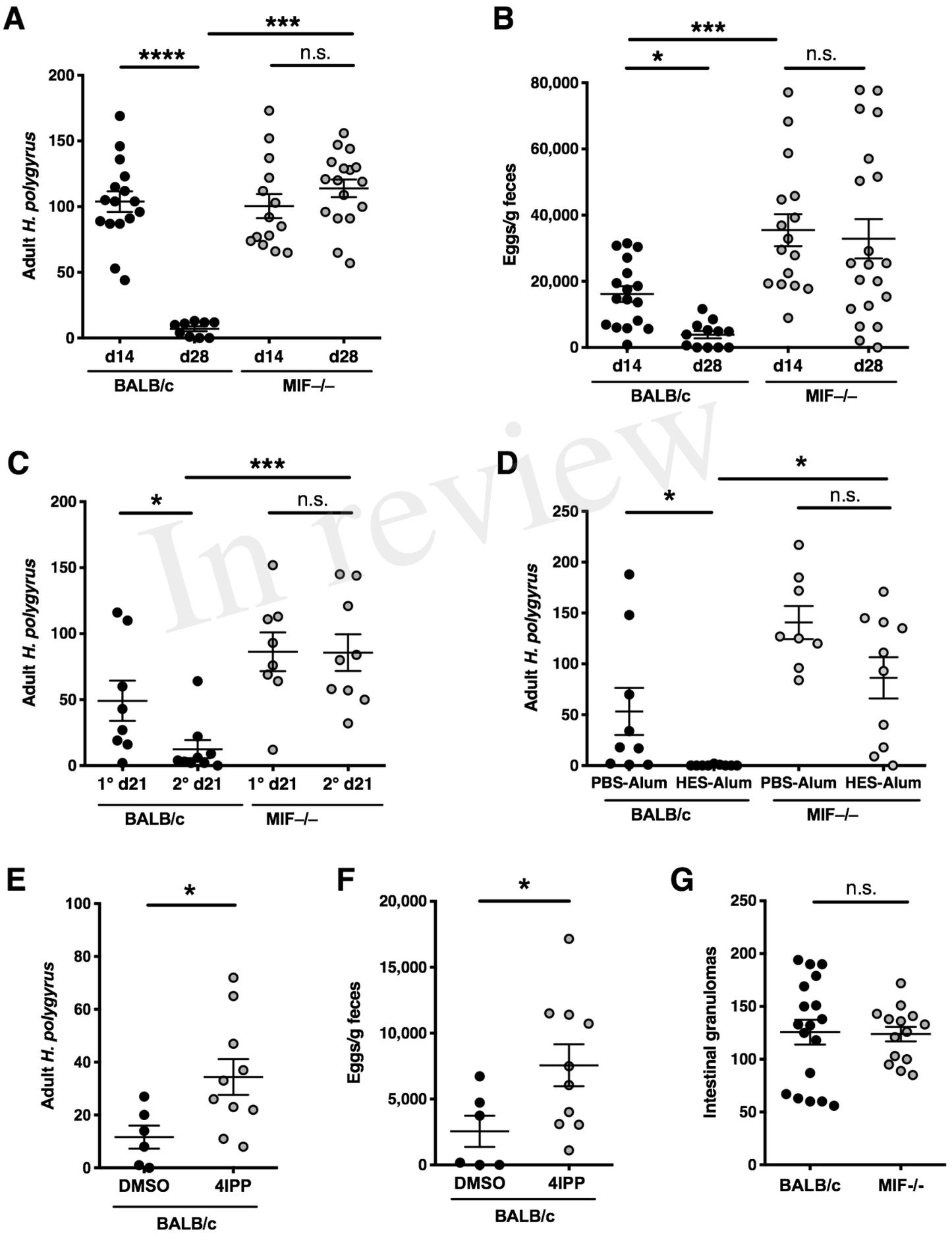
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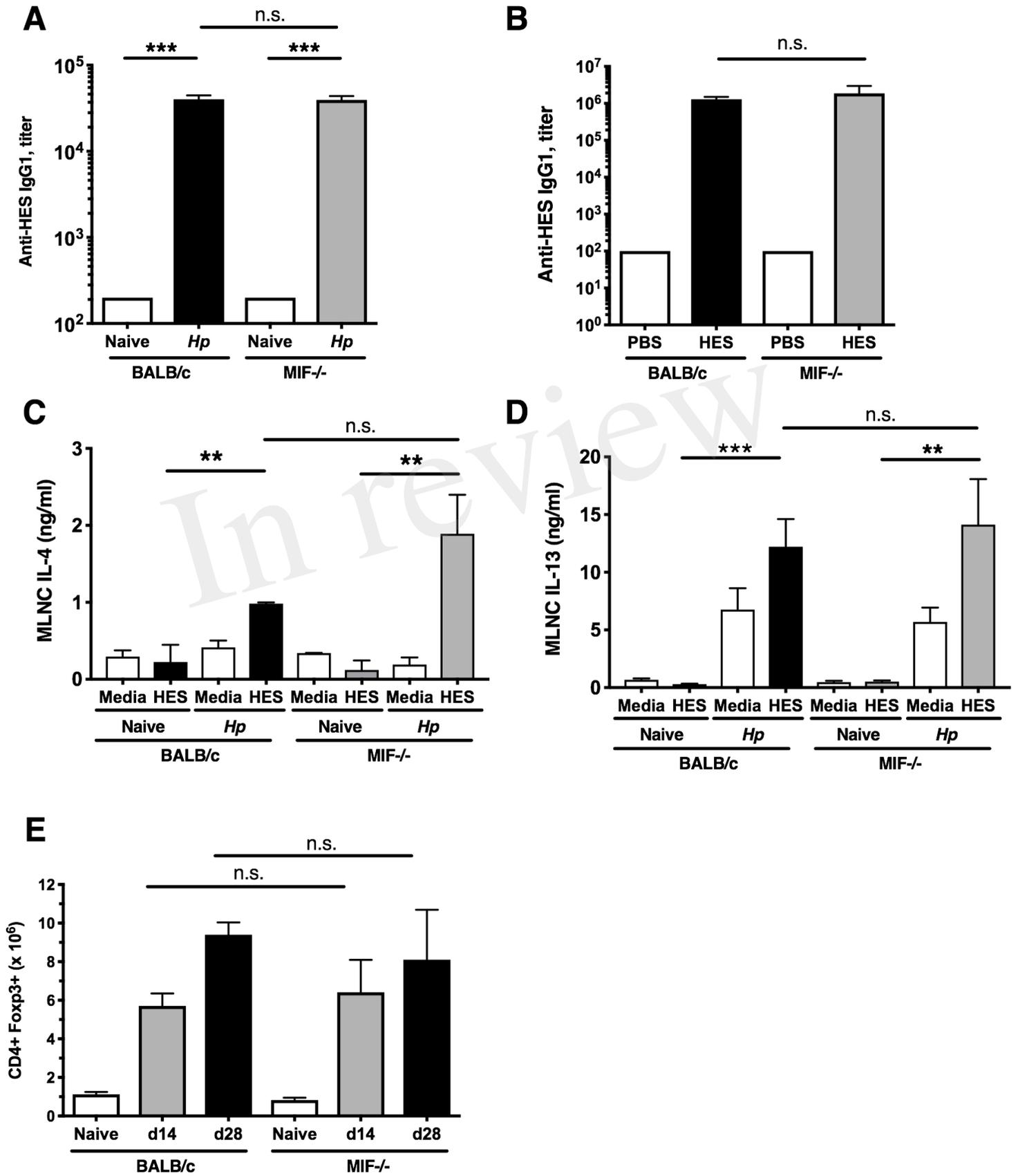
943 **Figure 8 - Myeloid cell expression of STAT3 is required for optimal Arginase-1 expression and**  
944 **for secondary immunity to *H polygyrus***

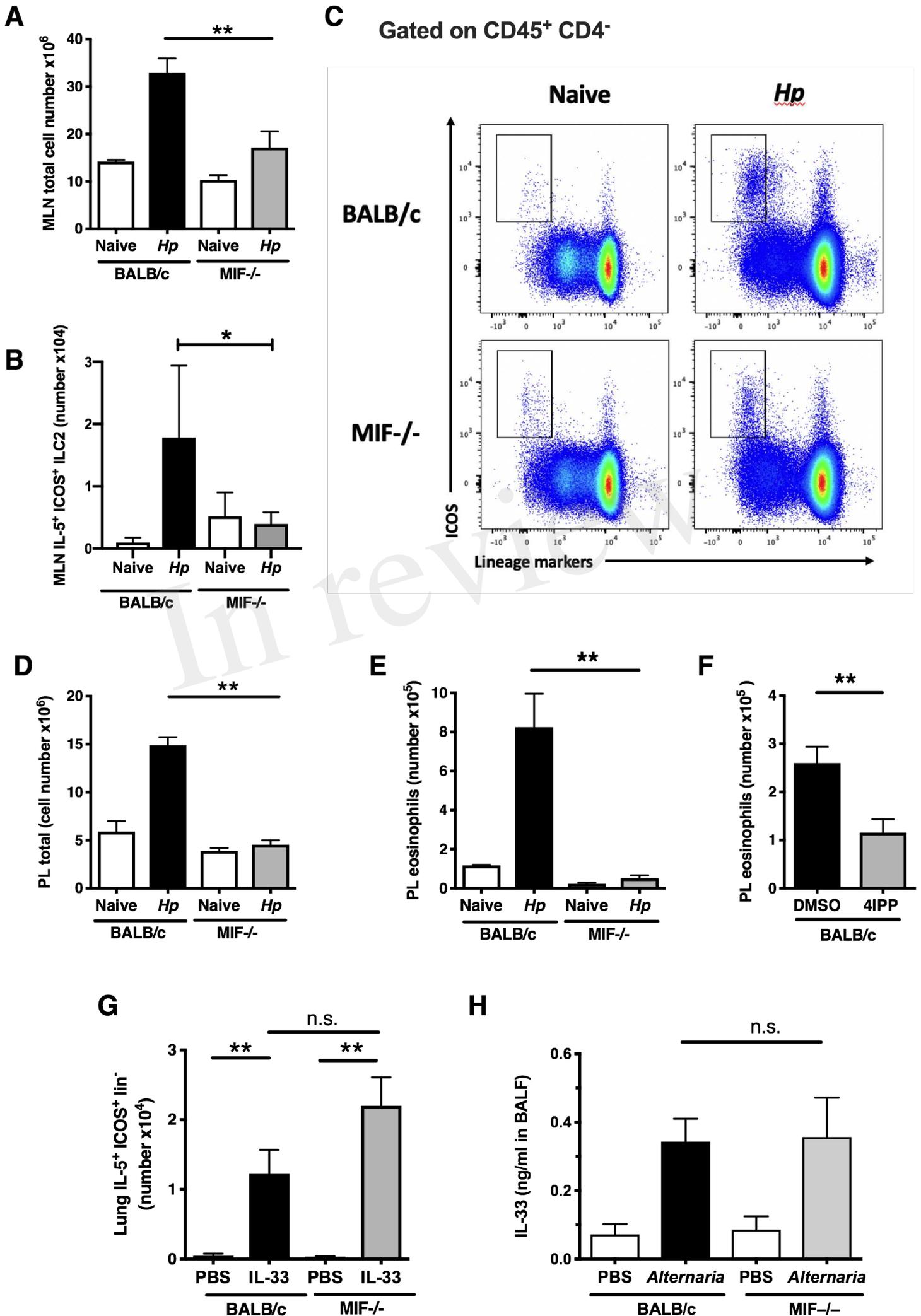
- 945 **A.** Peritoneal macrophages were recovered from conditional STAT3 gene deleted and control  
946 mice, with genotypes of STAT3<sup>fl/fl</sup>xLysM<sup>Cre/-</sup> and STAT3<sup>fl/fl</sup>xLysM<sup>-/-</sup> respectively, and stained  
947 for surface CD11b and F4/80, together with intracellular Arginase -1. Staining was evaluated  
948 by flow cytometry. Data are pooled from three independent experiments. \* = p<0.05.
- 949 **B.** Expression of Chil3 within the same macrophage populations. Data are pooled from three  
950 independent experiments. n.s. = not significant.
- 951 **C** Peritoneal lavage fluids were recovered from the same animals as in **A** and **B**, and functional  
952 soluble arginase levels measured by an enzyme assay. \* = p<0.05.
- 953 **D.** Presence of Chil3 protein in the same lavage fluids as **C**. \* = p<0.05.
- 954 **E.** Myeloid cell-specific deletion of STAT3 impairs secondary immunity. LysM<sup>Cre</sup>xSTAT3<sup>fl/fl</sup>  
955 mice and STAT3<sup>fl/fl</sup> controls) were infected with *H. polygyrus* and infections cleared with  
956 pyrantel embonate. Mice were subsequently challenged with a secondary infection, or infected  
957 for the first time for primary controls, and adult worms counted in the small intestine at d21  
958 post infection. Results are combined from two experiments with similar results. n.s. = not  
959 significant, \* = p<0.05.

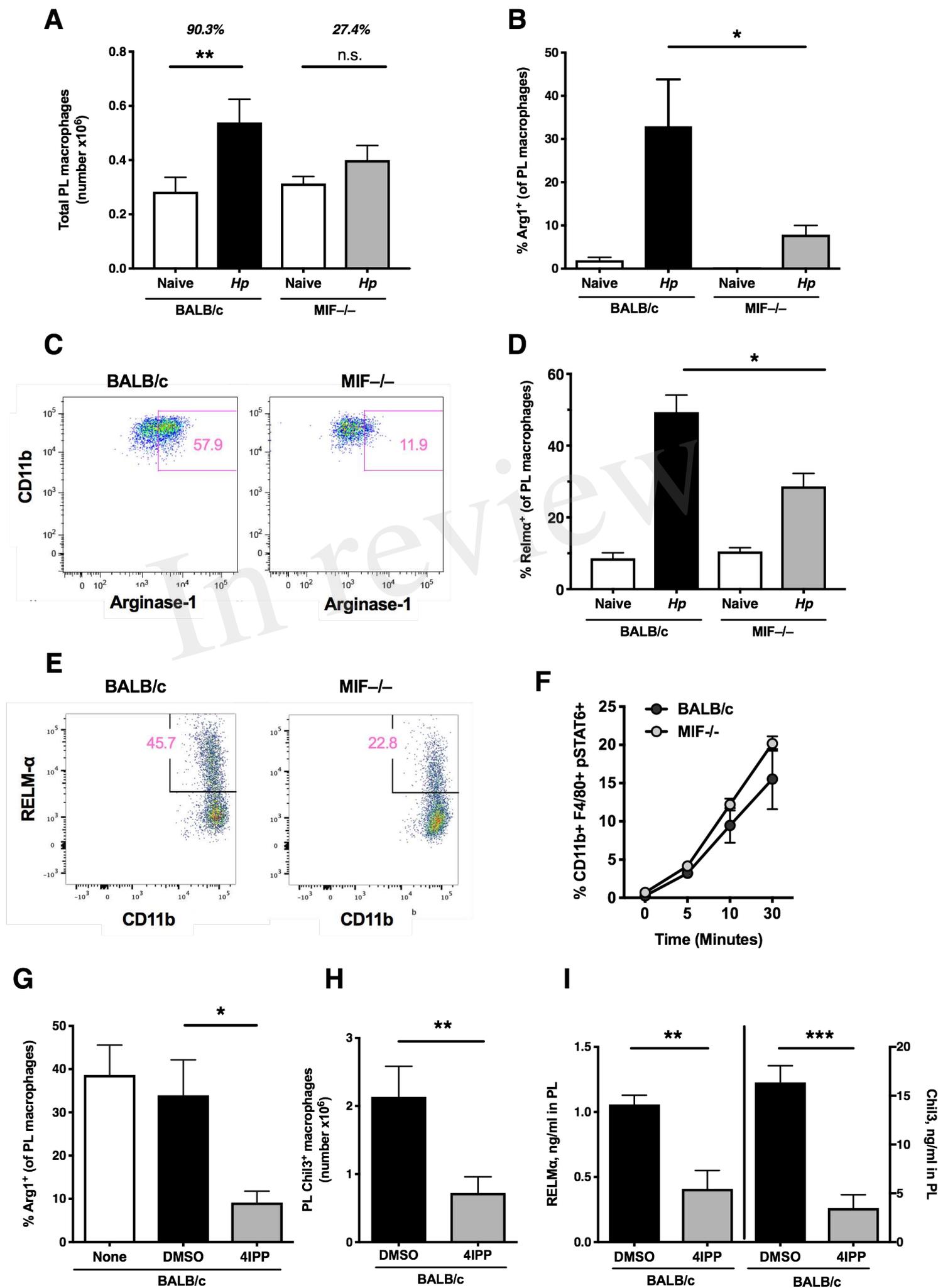
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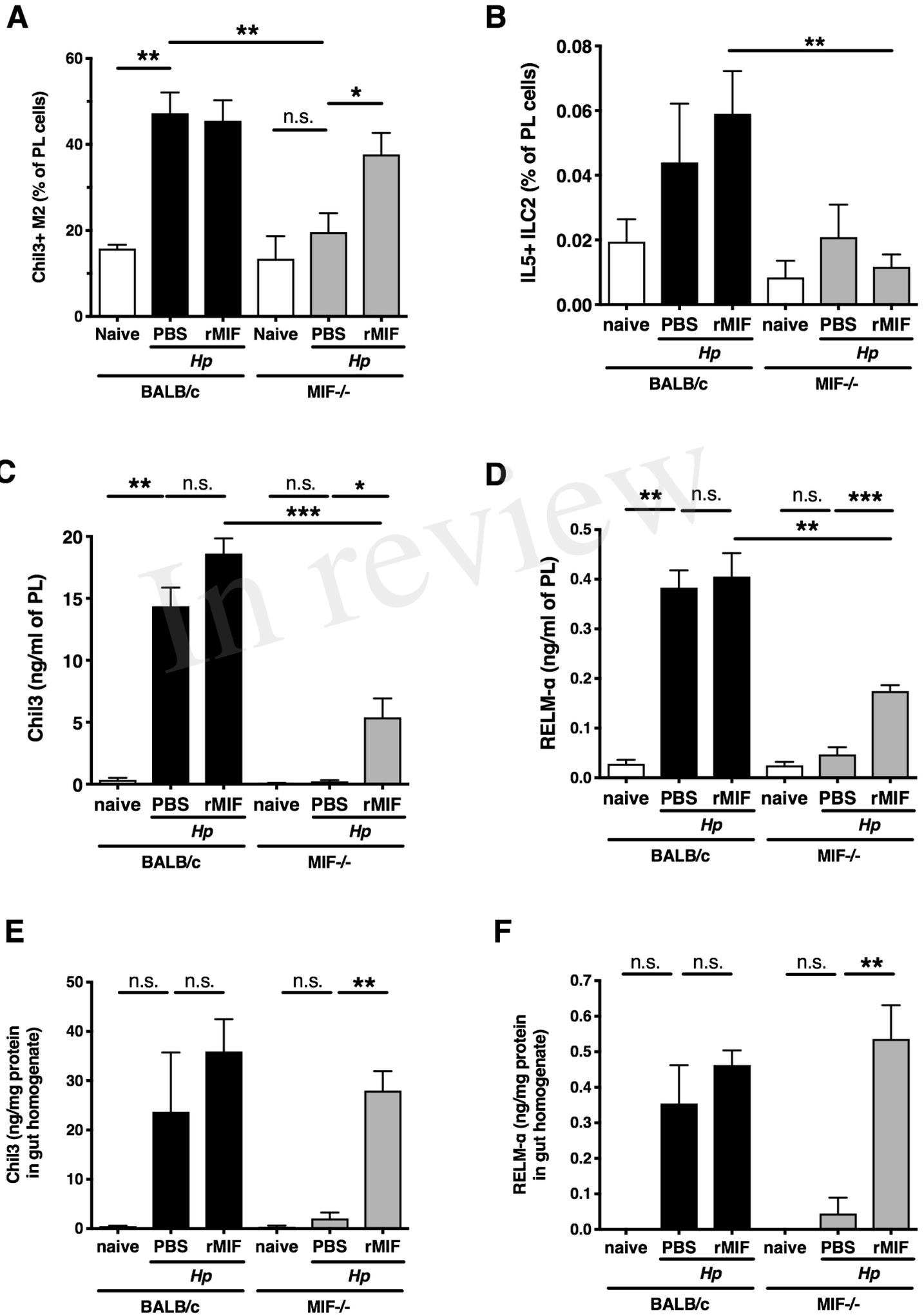
Filbey *et al.* Figure 1



Filbey *et al.*, Figure 2







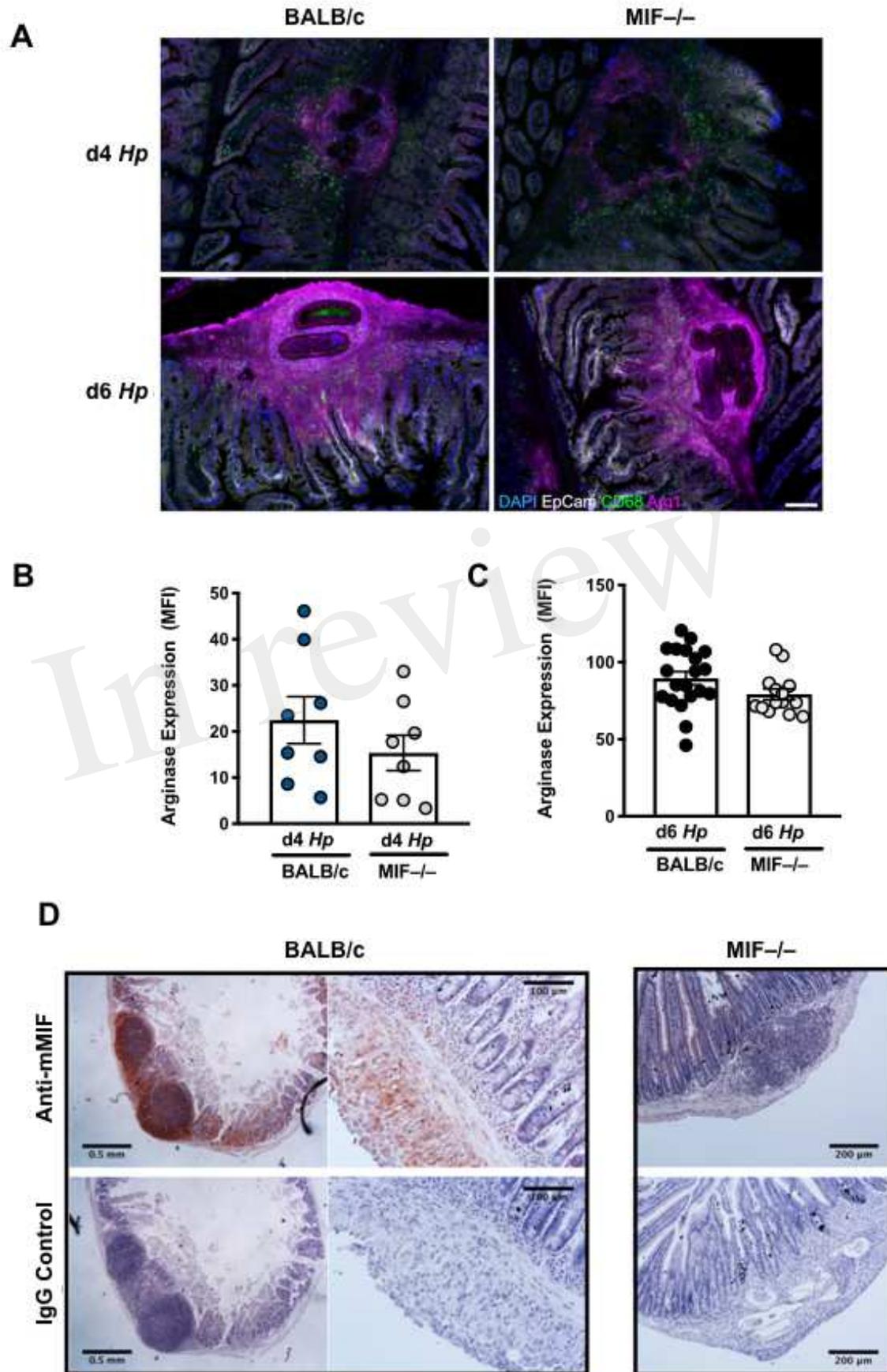
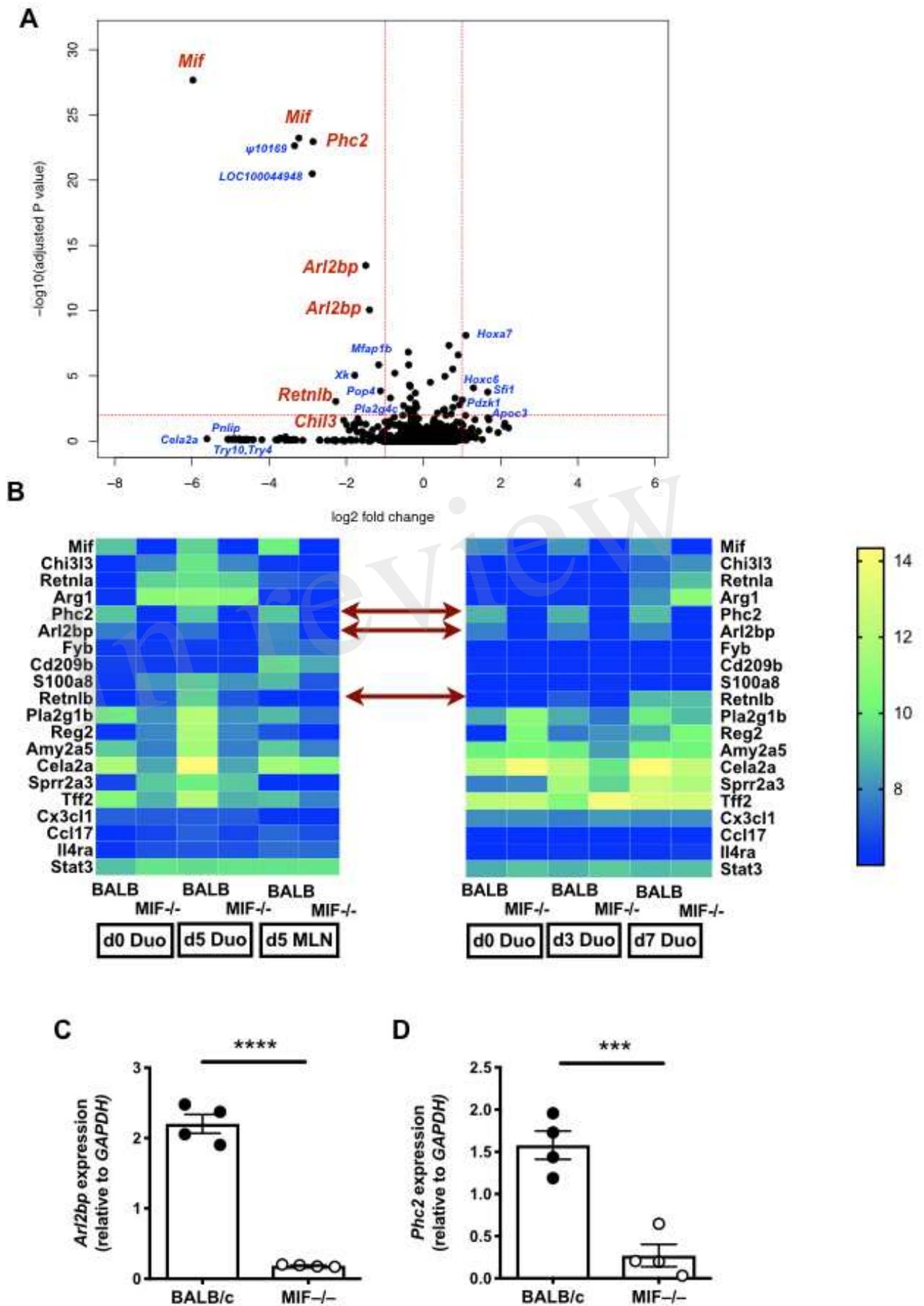
Filbey *et al.*, Figure 6

Figure 7.TIFF

Filbey et al. Figure 7



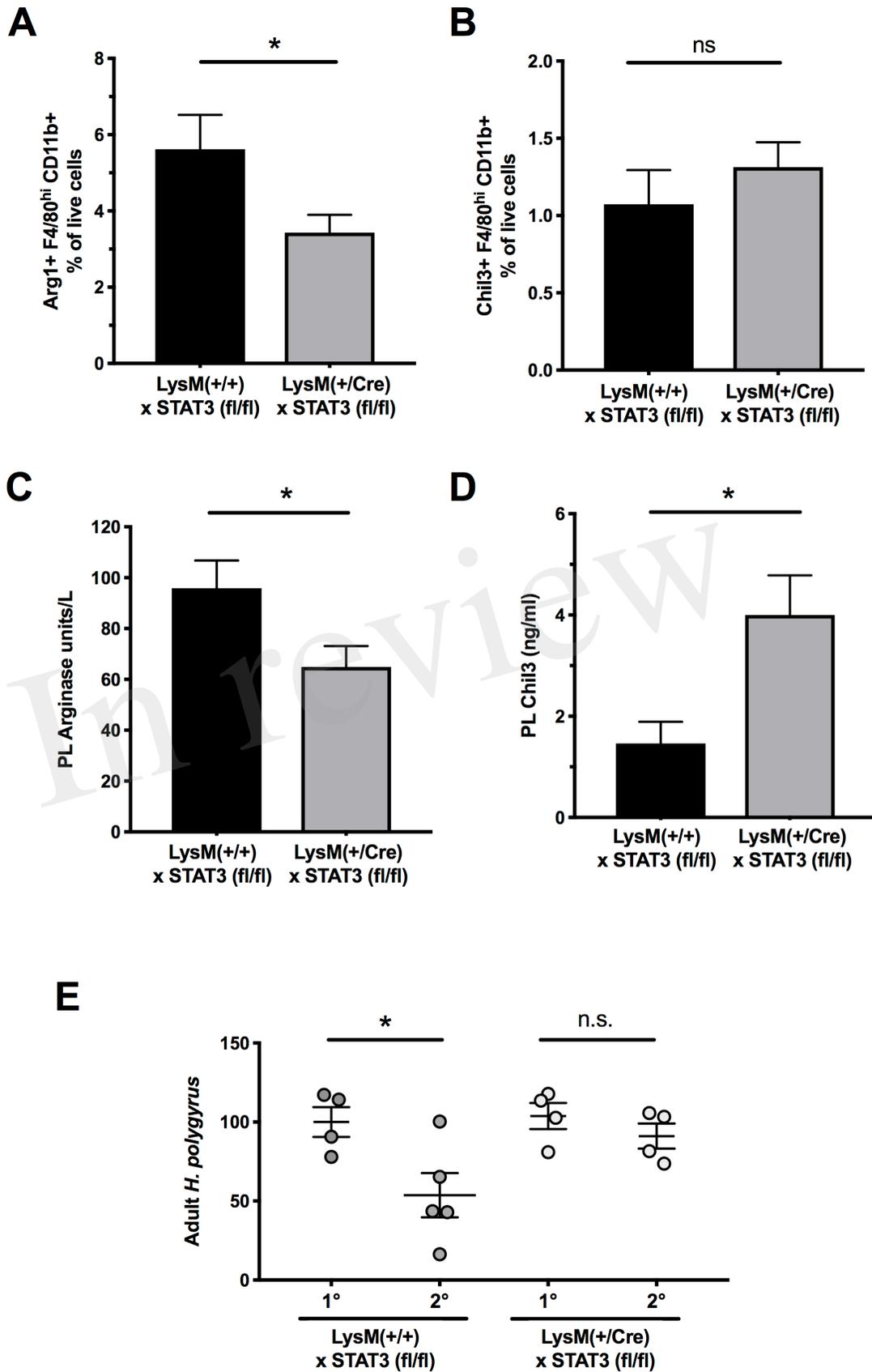
Filbey *et al.* Figure 8

Figure 9.TIFF

