Supporting Online Material for

Local macrophage proliferation, rather than recruitment from the blood, is a signature of Th2 inflammation.

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Materials and Methods

Animals, parasites and reagents.
All mice were bred and maintained in specific-pathogen free facilities at the University of Edinburgh. All work conformed to UK Home Office and institutional requirements. Experimental mice were age and sex matched, and C57BL/6 unless otherwise specified. C57BL/6 *Rag1*$^{-/-}$, C57BL/6 *Il4*$^{-/-}$ and BALB/c *Il4ra*$^{-/-}$ were produced and obtained as described previously (*17, 31*). Bone marrow chimeric mice were constructed by exposing anaesthetized C57BL/6 *Cd45.1* mice to a single dose of 9.5 Gy γ radiation while shielding the thorax, head and fore limbs with a 2-inch lead screen followed by i.v. injection of 6 x 10^6 donor bone marrow cells from congeneic *Cd45.2* mice. Chimeric mice were left for 8 weeks before further experimental manipulation. *L. sigmodontis* life cycle was maintained and infective third-stage larvae (L3) obtained as described elsewhere (*18*). Adult *B. malayi* worms were obtained from infected gerbils (TRS Laboratories) by peritoneal lavage (*17*). Reagents were from Sigma-Aldrich (Poole, UK) unless otherwise specified.

L. sigmodontis infection and thioglycollate-induced sterile pleurisy.
Mice were infected subcutaneously with 25 *L. sigmodontis* L3. Analysis of pleural cavity cells was performed at time-points between day 4 to 15 post-infection as indicated. For induction of classical inflammation in the pleural cavity, mice were given intra-thoracic injection of 200 μl 4% BBL Brewers modified thioglycollate medium (BD Europe, Oxford, UK). Unless otherwise specified, analysis post-thioglycollate injection was performed at day 3.

Implant of *B. malayi* worms and incisional wound model.
Mice were surgically implanted with one male and four female adult *B. malayi* worms into the peritoneal cavity and the wound sutured (*17*). Peritoneal surgery without parasite implantation was used to control for the surgical procedure and also provides a model of incisional wounding characterized by IL-4Rα-dependent M2 activation of the peritoneal population (*17*). Analysis of peritoneal exudate populations was performed at day 3, 6 and 10 post-surgery.

Long acting IL-4 complex (IL-4c).
Treatment of mice with a complex of IL-4 and anti-IL-4 mAb results in the slow continuous release of cytokine (*19, 32*). IL-4c consisted of a 2:1 molar ratio of recombinant mouse IL-4 (Peprotech, London) and anti-IL-4 mAb (clone 11B11; BioXcell, NH). Mice were injected i.p. with IL-4c containing 5 μg of IL-4 and 25 μg of 11B11, or PBS vehicle control on days 0 and 2. For co-administration experiments, mice were given thioglycollate by intra-thoracic (200 μl) or intra-peritoneal (800 μl) injection on day 0 immediately prior to the first dose of IL-4c. In all experiments, analysis of tissue inflammation was performed on day 4.

BrdU labelling of proliferating cells in vivo.
Mice were injected i.p. with 100ul of 10mg/ml BrdU in Dulbecco's PBS 3hr before experimental end-point.

Isolation of cells from the peritoneal cavity, pleural cavity and liver.
Following sacrifice, pleural or peritoneal cavity exudate cells were obtained by washing of the cavity with RPMI 1640 containing 2mM L-glutamine, 200U/ml Penicillin, 100 μg / ml Streptomycin (Invitrogen, Paisley, UK) and 0.2% normal mouse serum (AbD Serotec, Kidlington, UK). Worm burden in the lavage fluid of *L. sigmodontis* infected mice was determined by counting under a stereo-microscope. Erythrocytes were removed by incubating with red blood cell lysis buffer. Mononuclear liver cells were obtained as described previously (*33*). Cellular content of the cavities and organs was assessed by cell counting using a Casy TT cellcounter (Roche, Burgess Hill, UK) in combination with multi-color flow-cytometry.

Flow cytometry.
Equal numbers of cells or 20 μl of blood were stained for each sample. Blood samples were mixed and washed with Hank's buffered saline solution containing 2mM EDTA (Invitrogen). Cells were stained with LIVE/DEAD® (Invitrogen). All samples were then blocked with 5 μg/ml αCD16/32 (2.4G2; produced in-house) and normal mouse serum (1:20) in FACS buffer (0.5% BSA, 2mM EDTA in Dulbeccos PBS) before surface staining on ice with antibodies to F4/80 (BM8), Siglec-F (E50-2440), Ly-6C (AL-21 or HK1.4), Ly-6G (1A8), Gr-1 (RB6-8C5), CD11b (M1/70), CD11c (N418), MHCII (M5/114.15.2), CD19 (eBio1D3), CD4 (GK1.5), CD3 (17A2) CD45.1 (A20) or CD45.2 (104) (Ebioscience, Hatfield, UK; Biolegend, Cambridge, UK; or BD Europe). Anti-Gr-1 antibody was used to detect both Ly-6C^{high} monocytes and Ly-6G^{high} neutrophils, which were discriminated as Gr-1^{int} or Gr-1^{high}, respectively. Alternatively, antibodies specific to the individual Ly-6 molecules were used where specified. Erythrocytes in blood samples were lyzed using FACSlysing solution (BD Europe).

For detection of intracellular RELMα during *L. sigmodontis* infection time-course, cells were cultured in vitro with 1:1000 Golgi Plug (BD Europe) in X-VIVO 15 media (Lonza Biologics, Slough, UK) supplemented with 2 mM L-glutamine for 4 hrs. All subsequent figures depict direct *ex-vivo* staining since culture does not greatly enhance detection (data not shown). Following surface staining, cells were fixed with 2% paraformaldehyde in PBS for 10 min at room temperature, permeabilized with Permwash (BD Europe) then stained with anti-Ym1/2 (Stemcell Technologies, Grenoble, FR), RELMα (R and D systems, Abingdon, UK) or polyclonal rabbit IgG control (Sigma) followed by zenon anti-rabbit reagent (Invitrogen). For detection of Ki67 and BrdU incorporation, cells were stained for surface markers then fixed and permeabilized using FoxP3 staining buffer set (Ebioscience). Cells were then stained with Ki67 (B57) set or anti-BrdU (B44) FastImmune^{TM} for 30 min at room temperature, or incubated first with or without DNase for 30 mins at 37^{o}C before staining with anti-BrdU antibody (B44) (BD Europe). Expression of RELMα and Ki67 was determined relative to isotype control staining. Incorporation of BrdU was determined relative to staining on cells from mice injected with PBS rather than BrdU, or on non-DNase treated cells.

Depletion of blood monocytes.

Mice were injected daily i.v. with 200 μl CL-loaded liposomes or PBS at time-points described in the figure legends. Following thioglycollate injection, PBS was injected on days that liposomes were not. Pleural cavity populations were analyzed on day 10 post-nematode infection or day 3 following thioglycollate injection. Monocyte depletion was assessed by flow-cytometric analysis of peripheral blood samples throughout these periods. Liposomes were generated as previously described (*34*) using phosphatidylcholine (Lipoid GmbH, Ludwighafen, Germany) and cholesterol. Clodronate was a gift from Roche Diagnostics GmbH (Mannheim, Germany).

Quantification of gene expression.

RNA was extracted from mediastinal and parathymic lymph nodes using RNeasy Lipid tissue mini kit (Qiagen, Crawley, UK) according to manufacturer's instructions. cDNA was generated using oligo dT primers (Roche Diagnostics Ltd., Burges Hill, UK) and Molony murine leukemia virus reverse transcriptase (Bioline, London, UK). Quantitative PCR was performed using SYBR Green PCR mix and a LightCycler 480 machine (Roche Diagnostics, Burges Hill, UK) and the following primer pairs: β-actin (5'-TGGAATCCTGTGGCATCCATGAAAC-3' and 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'), IL-13 (5'-CCTCTGACCCTTAAGGAGCTTAT-3' and 5'-CGTTGCACAGGGGAGTCT-3'), IL-5 (5'-ACATTGACCGCCAAAAAGAG-3' and 5'-CACCATGGAGCAGCTCAG-3'), and IFNγ (5'-GGAGGAACTGGCAAAAGGAT-3' and 5'-TTCAAGACTTCAAAGAGTCTGAGG-3'). Relative quantities of nucleic acid material were determined against an internal standard curve and standardized against expression of β-actin.

Statistical analysis.

Statistical analysis was performed using GraphPad PRISM 4. Where applicable, data were analyzed by unpaired two-tailed *t* test. One or two-way ANOVA with Tukey or Bonferroni post-test, respectively, or Kruskal-Wallis test with Dunn's post-test were used for comparisons of more than two groups. If required, data were first log transformed to achieve normal distribution.

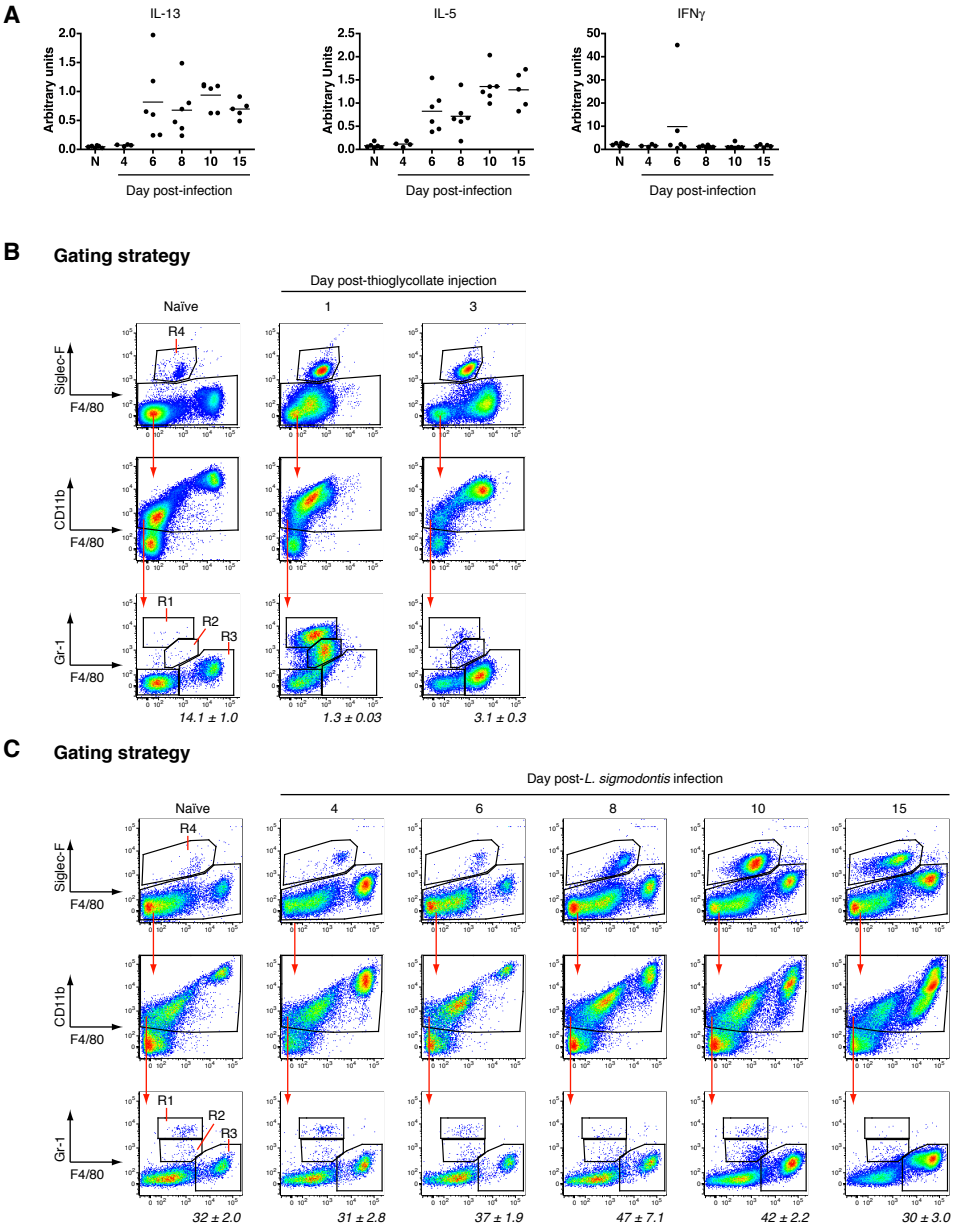

Fig. S1. Kinetics of the immune response during *L. sigmodontis* infection, and gating strategy for pleural inflammatory cells. (**A**) Expression of cytokines in the mediastinal and parathymic lymph nodes following infection with *L. sigmodontis*, determined by quantitative PCR and normalized against expression of β-actin. (**B**) Gating strategy defining eosinophils (R4), neutrophils (R1), Gr-1^{int}/Ly-6C^{high} monocytes (R2), and F4/80^{+} macrophages (R3) in the pleural cavity following intra-thoracic injection of thioglycollate or (**C**) after *L. sigmodontis* infection. Numbers in italics are mean fluorescent intensity (*10^3) of staining for F4/80 within the macrophage gate R3. (A-C) Data are representative of 2 independent experiments, with 4-6 mice per group. Data presented are from the same experiments as those shown in Fig. 1 A-D. N = naïve.

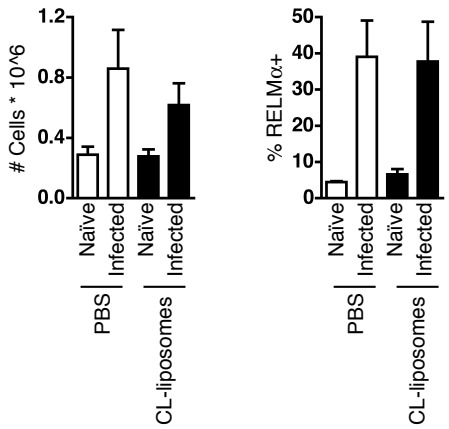

Fig. S2. Pleural macrophages accumulate during *L. sigmodontis* infection independently of blood monocytes. Number of F4/80^{+} macrophages in the pleural cavity and the proportion producing RELMα on day 10 post-infection from naïve or *L. sigmodontis* infected mice injected i.v. with CL-liposomes (black bars) or PBS (white bars) daily from day 2 to 5 post-infection. Data are representative of two independent experiments. Graphs present mean ± SEM of 4-8 mice per group.

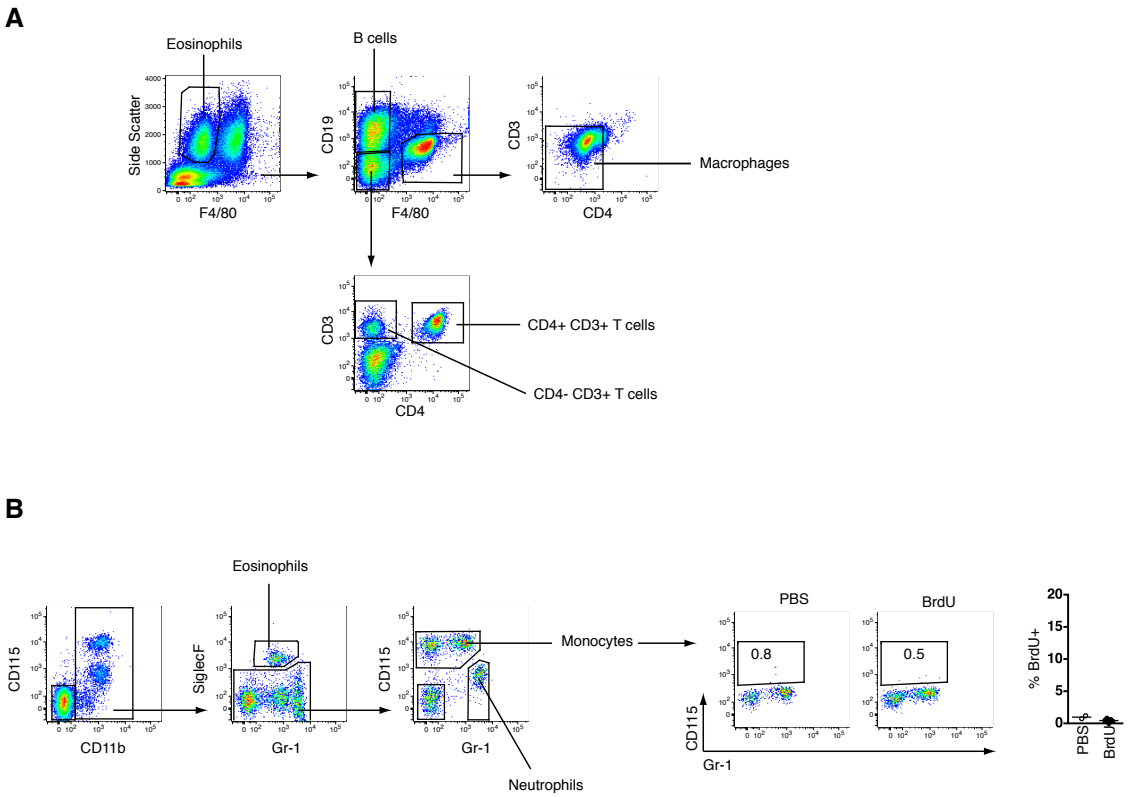

Fig. S3. Gating schemes for analysis of BrdU incorporation by pleural macrophages or blood monocytes. (**A**) Gating strategy used to define F4/80^{+} pleural macrophages for analysis of BrdU incorporation specifically to ensure no contamination by proliferating lymphocytes. (**B**) The proportion of blood monocytes incorporating BrdU in vivo on day 10 post-*L. sigmodontis* infection. Mice were given BrdU or a control injection of PBS 3 hours prior to analysis. Monocytes gated as shown. Analysis restricted to infected mice only, with 2 PBS and 5 BrdU treated mice per group. Data are representative of 2 independent experiments.

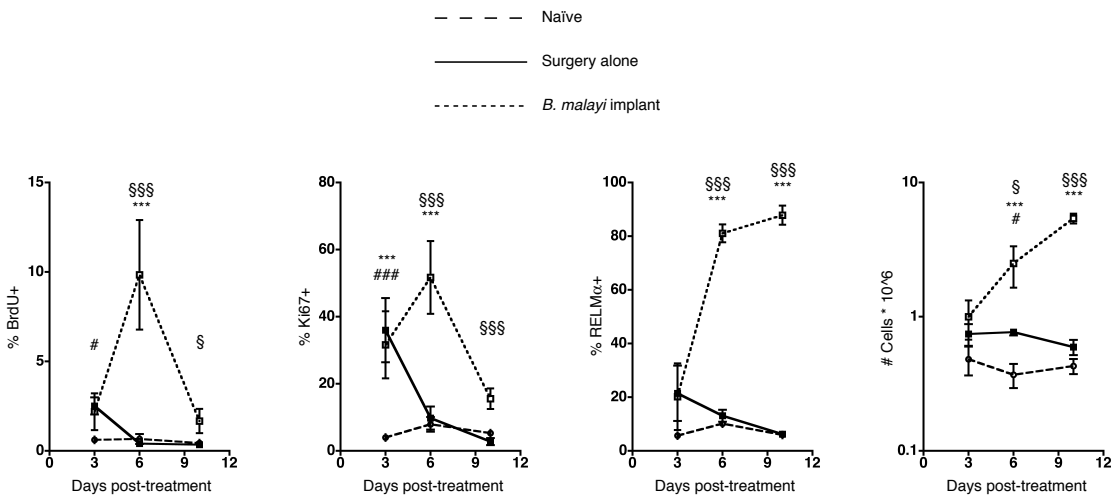

Fig. S4. Macrophage proliferation is characteristic of type-2 inflammation. Total number and proportion of peritoneal F4/80^{+} macrophages positive for BrdU, Ki67 or RELMα at day 3, 6 and 10 following surgical implantation of adult *B. malayi* worms into the peritoneal cavity, peritoneal surgery alone or in naïve mice. Data are presented as mean ± SEM of 4-5 mice per group. Representative of two independent experiments. #P <0.05, and ###P <0.001, for surgery alone compared to naïves, ***P <0.001, for parasite implant compared to naïves, §P <0.05 and §§§P <0.001, for parasite implant compared to surgery alone, as determined using two-way ANOVA, with Bonferroni post-test for multiple comparisons.

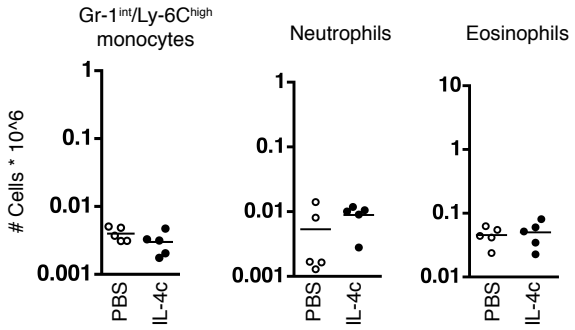

Fig. S5. Neither monocytes, neutrophils nor eosinophils are recruited following treatment with IL-4c. Total number of inflammatory Gr-1^{int}/Ly-6C^{high} monocytes, neutrophils and eosinophils in the peritoneal cavity exudate population on day 4 after peritoneal injection of PBS or IL-4c on days 0 and 2. Representative of 5 independent experiments.

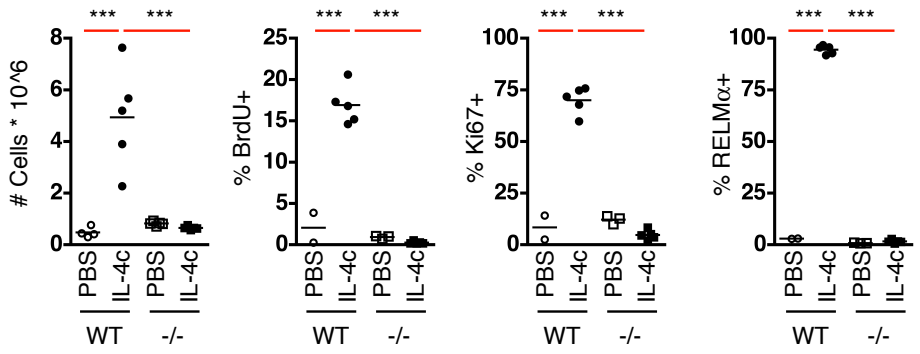

Fig. S6. IL-4c has no activity in *Il-4ra*$^{-/-}$ mice. Total peritoneal F4/80^{+} macrophages and the proportion staining positive for BrdU, Ki67 or RELMα from BALB/c mice (WT) or *Il4ra*$^{-/-}$ mice (-/-) on day 4 after peritoneal injection of PBS or IL-4c on days 0 and 2. Data are representative of 3 independent experiments. ***P <0.001 as determined by one-way ANOVA with Tukey's post-test for multiple comparisons.

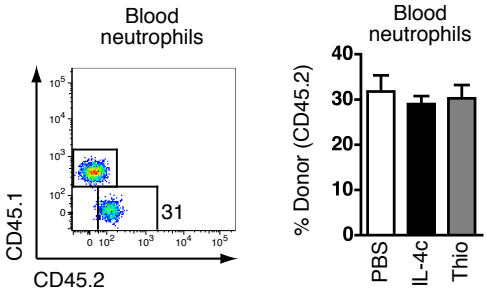

Fig. S7. Chimerism of blood neutrophils. Flow cytometric analysis of chimerism of blood neutrophils in *Cd45.1* congenic mice given CD45.2^{+} bone marrow after partial-body irradiation, and subsequently treated with PBS or IL-4c i.p., or intra-thoracic thioglycollate at week 8 post-reconstitution. Representative staining for CD45.1 and CD45.2 on blood neutrophils using gating strategy shown in fig. S3B. Graphs present mean ± SEM of 4 mice per group. Data are from the same experiment as results in Fig. 3D, and are representative of 2 independent experiments.

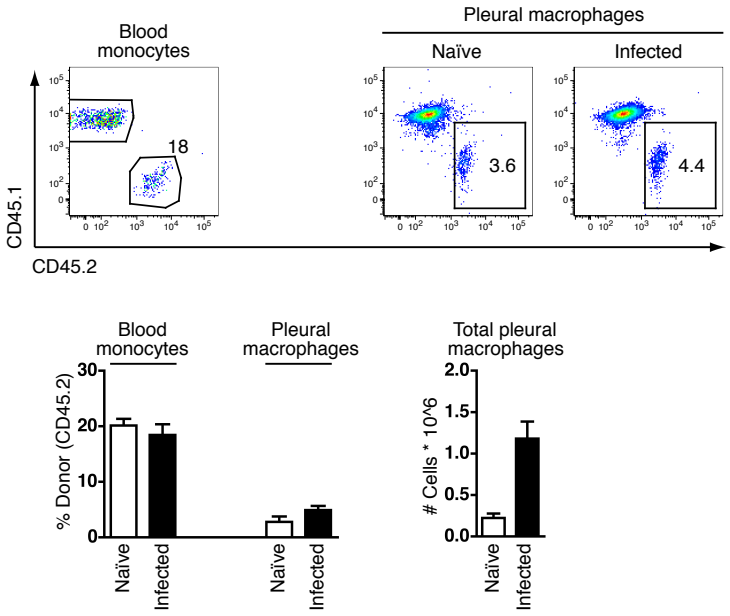

Fig. S8. Accumulation of pleural macrophages during *L. sigmodontis* infection is independent of recruitment of bone marrow derived cells. Flow cytometric analysis of chimerism of blood monocytes and pleural macrophages and number of pleural macrophages in *Cd45.1* congenic mice given CD45.2^{+} bone marrow cells after partial-body irradiation, and subsequently infected with *L. sigmodontis* at week 11 post-reconstitution. Representative CD45.1 and CD45.2 staining gated on CD11b^{+} CD115^{high} blood monocytes or F4/80^{+} pleural macrophages. Graphs depict mean ± SEM of 4 mice per group.

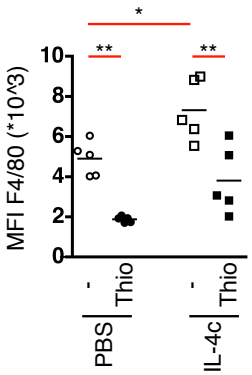

Fig. S9. Co-administration of thioglycollate with IL-4c elicits a population of F4/80^{low} macrophages. Geometric MFI of F4/80 staining upon peritoneal macrophages from mice treated i.p. with thioglycollate on day 0 and IL-4c or PBS vehicle control on days 0 and 2. Control mice received PBS or IL-4c without thioglycollate (-). Analysis was performed on day 4. Data are compiled from flow cytometry shown in Fig. 3A and Fig. 4, which were performed and analyzed simultaneously. Data are representative of 2 independent experiments. **P* <0.05 and ****P* <0.001 as determined by one-way ANOVA with Tukey's post-test for multiple comparisons.

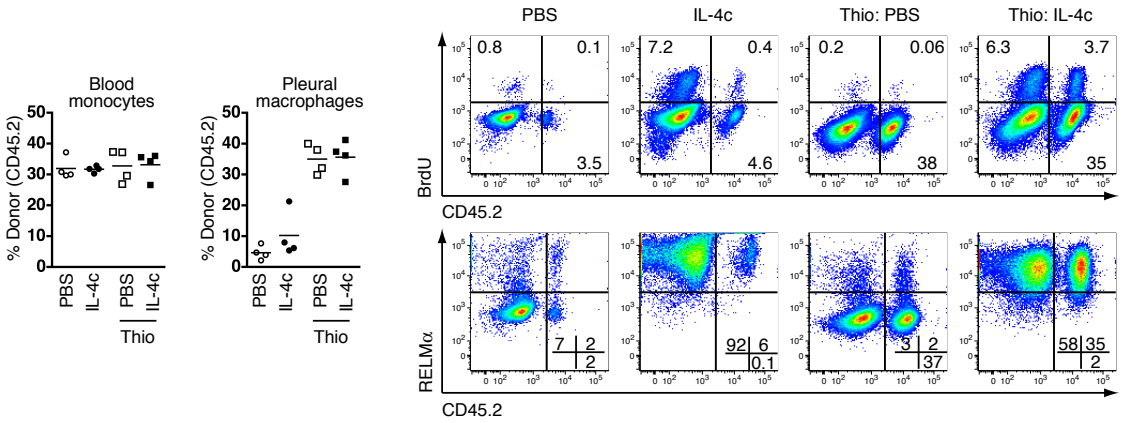

Fig. S10. IL-4 drives proliferation of recruited inflammatory macrophages. Flow cytometric analysis of chimerism of blood monocytes and pleural macrophages and number of pleural macrophages in *Cd45.1* congenic mice given CD45.2$^+$ bone marrow after partial-body irradiation, and subsequently injected simultaneously with intra-thoracic thioglycollate and PBS or IL-4c i.p. at week 8 post-reconstitution. Control chimeric mice received PBS or IL-4c i.p. alone. Analysis was performed at day 4 post-thioglycollate injection. Representative staining for CD45.2 and BrdU, or CD45.2 and RELMα gated on F4/80$^+$ pleural macrophages. Data are from a single experiment using 4 mice per group. These data show that the proliferating pleural macrophages found in mice treated with both thioglycollate and IL-4c are recruited to the tissues from bone marrow-derived precursors, since they exhibit a frequency of CD45.2 chimerism that matches blood monocytes and is distinct from resident pleural macrophages from animals not injected with thioglycollate.