Schistosomiasis, one of the most important neglected tropical diseases worldwide, is caused by flatworms (blood flukes or schistosomes) that live in the bloodstream of humans. The hepatointestinal form of this debilitating disease results from a chronic infection with *Schistosoma mansoni* or *Schistosoma japonicum*. No vaccine is available to prevent schistosomiasis, and treatment relies predominantly on the use of a single drug, praziquantel. In spite of considerable research effort over the years, very little is known about the complex *in vivo* events that lead to granuloma formation and other pathological changes during infection. Here we use, for the first time, a lentivirus-based transduction system to deliver microRNA-adapted short hairpin RNAs (shRNAmir) into the parasite to silence and explore selected protein-encoding genes of *S. mansoni* implicated in the disease process. This gene-silencing system has potential to be used for functional genomic-phenomic studies of a range of socioeconomically important pathogens.
chistosomiasis is among the most important neglected tropical diseases worldwide, affecting ~200 million people globally and causing 300,000 deaths per annum. The hepatointestinal form of this debilitating disease is usually caused by a chronic infection with *S. mansoni* or *S. japonicum* (blood flukes). No vaccine is available, and treatment relies on the use of one drug (praziquantel), to which resistance is emerging. Of the three main species of schistosome that infect people, *S. mansoni* is widespread throughout Africa, South America and the Caribbean. Through a complex aquatic life cycle, *S. mansoni* is transmitted (via skin penetration) from an infected, aquatic snail (* Biomphalaria spp.*) to humans. Adult worms dwell in hepatic and intestinal vessels, where they release eggs that become embedded in the liver or intestinal wall, and trigger immune-mediated granuloma formation and associated clinical complications, such as periportal fibrosis and hypertension.

Although some immunopathological changes linked to the complications, such as periportal fibrosis and hypertension, 2. Nonetheless, recent evidence has shown great promise for the use of gene knockdown for subsequent phenotypic assessment in vivo. Various tools, such as RNA interference (RNAi) using double-stranded RNA or short interfering RNA (siRNA), have been used to investigate the functions of single genes; however, some techniques employed to date can have limitations, such as off-target (that is, nonspecific) effects and an inadequate persistence of gene knockdown for subsequent phenotypic assessment in vitro or in vivo in the host(9). These issues are compounded by the challenges of consistently producing sufficient amounts of appropriate parasite stages for functional genomic analyses.

Nonetheless, recent evidence has shown great promise for the use of a lentivirus-based transduction system for specific and persistent gene knockdown in mammalian cells, with the potential of overcoming most of the disadvantages of previous knockdown methods.

Here we show how lentivirus transduction can be used to achieve specific and persistent knockdown of selected genes (*omega-1*, *ipse* and/or *kappa-5*) of *S. mansoni*, implicated in egg-induced granulomatous responses in the mammalian host. This study allows, for the first time, the functional genomic–phenomic exploration of chistosomiasis mansoni in vivo.

### Results

**Lentivirus transduction.** First, we transduced *S. mansoni* eggs with lentiviral constructs encoding microRNA-adapted short hairpin RNAs (shRNAmirs) (Fig. 1a). Ten days after transduction with lentiviral particles, both regions of the *mCherry* gene and regions encoding individual shRNAmirs were detected in the genomic DNAs of treated eggs, whereas neither of these regions was found in DNA from unexposed eggs (Fig. 1b), indicating successful delivery of viral DNA into the nucleus of the host cell. Southern blot hybridization confirmed proviral integration into the genome of *S. mansoni* (Fig. 1c). Moreover, *mCherry* was transcriptionally active under the control of the cytomegalovirus (CMV) promoter, as transcripts were detected in complementary DNA from virus exposed but not from unexposed eggs (Fig. 1b). Although attempts to detect fluorescence and assess transduction efficiency in transduced eggs were impaire by autofluorescence of the eggshell (not shown), results demonstrated successful lentiviral transduction and CMV-driven transgene expression in *S. mansoni* eggs.

shRNAmirs silence transcription in *S. mansoni in vitro*. Before testing in *S. mansoni* eggs, we assessed the functionality of seven shRNAmirs in mammalian (COS7) cells expressing cDNA of the target. Treatment with shRNAmir-511, shRNAmir-557 or shRNAmir-558, targeting the *omega-1* gene, showed a 29–34% decrease in transcription compared with ‘empty-vector’ (EV) control samples; the transcription of the *ipse* and *kappa-5* genes was consistently reduced by 33–42% (shRNAmir-195 or shRNAmir-384) and 28–34% (shRNAmir-616 or shRNAmir-638), respectively, following treatment (Fig. 2a). The subsequent transduction of *S. mansoni* eggs with lentivirus encoding shRNAmir-557 and targeting transcripts of *omega-1* led to a 45–85% decrease in transcription compared with EV control samples in three independent experiments (Fig. 2b); transcription in EV-treated eggs did not differ from that of untreated control samples. This result shows, for the first time, that shRNAmirs are functional in *S. mansoni* and can be used for RNAi. Next, we assessed gene knockdown in *S. mansoni* eggs using a combination of two viruses encoding different shRNAmir sequences that targeted *omega-1* (shRNAmir-511 + shRNAmir-557), *ipse* (shRNAmir-195 + shRNAmir-384) or *kappa-5* (shRNAmir-616 + shRNAmir-638). Three days after viral transduction, a 50–60% reduction in transcription was recorded for all shRNAmir treatment groups compared with EV controls (Fig. 2c); other results showed that the knockdown effect on target gene transcription persisted for at least 10 days following transduction (Fig. 2c). Importantly, lentiviral transduction and shRNAmir-induced knockdown of transcription had no effect on the maturation or vitality of miracidia in eggs (Supplementary Movie), providing the basis for an evaluation of transduced eggs in vivo in mice.

**Effect of knockdown on egg–induced pathology.** As *omega-1*, *ipse* (egg-secreted cytotoxic or immunomodulatory glycoproteins) and *kappa-5* (non-secreted protein) have been implicated in egg-induced responses, we investigated whether knockdown of genes encoding each of these egg proteins had an effect on the development of granulomata associated with pulmonary chistosomiasis induced in BALB/c mice following the injection of *S. mansoni* eggs into the tail vein. In this established experimental mouse model, eggs are transported to the lungs via the bloodstream and pass into the lung tissues where they become embedded and induce immune responses, leading to subsequent granuloma formation that peaks ~2 weeks after injection. In our investigation, there was a reduction in the numbers of dendritic cells (DCs), T-helper and B cells, as well as interstitial macrophages in the lungs of mice injected with *omega-1* knockdown eggs compared with those injected with eggs carrying the EV (Fig. 3a); the numbers were consistent with those of naive mice. The number of alveolar macrophages in the *omega-1* knockdown group was similar to that of the EV control mice, but elevated compared with naive mice (Fig. 3a). A slight decrease in infiltrating interstitial macrophages was also observed in *ipse* and *kappa-5* knockdown mice, but was not significantly different from the EV control mice (Fig. 3a). Interestingly, the numbers of eosinophil granulocytes infiltrating the lung were not affected by knockdown of any of the target genes of *S. mansoni* (Fig. 3a). Moreover, lung-associated neutrophil numbers were not altered in any of the treatment groups compared with naive control mice (Fig. 3a). The numbers of leukocyte infiltrates in the lung tissue did not differ significantly between wild type (WT) and EV-transduced, egg-injected control mice. Serum samples from mice that had been injected with WT or control virus-transduced eggs had an expected, significant increase in IgE levels, typical of *S. mansoni* infection; an increase was also seen in all egg-protein-knockdown groups of mice (Fig. 3b). Following a
soluble egg antigen (SEA)-specific re-stimulation of lung cell isolates (Fig. 4), all egg-injected mice responded with a characteristic, dominant Th2 cytokine pattern compared with the naive control group. However, no significant difference was observed in any of the knockdown groups compared with EV or WT egg-injected mice.

Finally, we assessed whether gene knockdown for any of the three egg proteins had an effect on granuloma formation in the lungs of mice (Fig. 3c). Knockdown of omega-1 in *S. mansoni* eggs led to a highly significant (*P < 0.001*) decrease in granuloma size compared with the EV and untreated, WT control groups, with mean ± s.e. of granuloma/egg ratios of 6.505 ± 0.963, 19.75 ± 1.685 and 16.63 ± 1.597, respectively. Furthermore, granuloma in the omega-1 knockdown group (6.505 ± 0.963) were significantly (*P < 0.01*) lower than those in ipse (10.31 ± 1.162) and kappa-5 (12.77 ± 1.648) knockdown groups. No significant differences in granuloma size were measured between mice injected with WT (19.75 ± 1.685) or control virus-treated eggs (16.63 ± 1.597; Fig. 3c). Granuloma found in the lungs of the kappa-5 knockdown group exhibited an increase in collagen deposition (Fig. 3d).

**Discussion**

Here we demonstrated lentiviral transduction of *S. mansoni* eggs for the delivery of a shRNA expression cassette. This approach probably overcomes some of the limitations of conventional RNAi (for example, by soaking or electroporation), including a lack of persistent knockdown over a longer period of time (which is required for *in vivo* application in animals) and possible off-target effects of RNAi triggers. Although double-stranded RNA and siRNA usually achieve knockdown for periods of up to 4 weeks in *S. mansoni in vitro*16,17, it has been shown that siRNA does not induce a long-term effect *in vivo* when schistosomes are re-introduced into the definitive host17. The findings of the present study show that the integration of a lentivirus encoding an RNAi-trigger expression cassette circumvents this limitation to achieve sustained knockdown in *S. mansoni in vivo* in mice. The results obtained using this method also indicate higher specificity of gene knockdown compared with conventional RNAi. In contrast to previous γ-retrovirus-based methods employed to deliver shRNAs to *S. mansoni*18–20, the lentiviral delivery system can transduce both arrested and dividing cells to maximize knockdown efficiency. Furthermore, the expression of shRNAs as artificial primary miRNAs with a Drosha and Dicer processing site has been shown to diminish the cytotoxic effect of a given short hairpin RNA (shRNA)21. The thermodynamic design of hairpins allows for a biased incorporation of the antisense strand into the RNA-induced silencing complex22. This approach decreases the risk of off-target effects induced by the sense (miRNA*) strand and enables off-target gene predictions to be made based on the antisense (miRNA) strand sequence. Moreover, the expression of shRNAs as shRNAmir can increase target gene knockdown by up to tenfold23. Therefore, the use of more efficient shRNAmir might also allow the application of weak promoters to minimize possible cytotoxicity linked to a saturation of the RNAi pathway24,25, and of low virus copy numbers to reduce the risk of adverse effects due to viral integration and promoter/enhancer interactions with endogenous genes, as shown previously for γ-retroviruses26. To avoid adverse effects resulting from an abundance of the RNAi trigger, shRNAmir expression levels need to be controlled. Such controlled expression of the RNAi trigger can be realized by...
Testing of gene knockdown in S. mansoni eggs in vitro

Assessment of gene knockdown in S. mansoni eggs in mice (Fig. 3)

Figure 2 | Validation of artificial microRNAs in COS7 cells and lentivirus-based transduction of S. mansoni eggs. (a) Validation of shRNA-mir lentivirus constructs in COS7 cells transfected with plasmids encoding omega-1, ipse or kappa-5. COS7 cells were either not transfected (C) or transfected with vector plasmids without the shRNA-mir stem-loop sequence (that is, empty-vector (EV)) or with vector plasmids containing gene-specific shRNA-mirs shRNA-mir-511, shRNA-mir-557 and shRNA-mir-558 (omega-1); shRNA-mir-195, shRNA-mir-384 (ipse), shRNA-mir-616 and shRNA-mir-638 (kappa-5) (labelled with numbers only). Transcription was assessed by qPCR after 48 h, using act-b as a reference gene. Kruskal–Wallis and Dunns post-hoc tests were used to establish statistical significance compared with the EV control: *P < 0.05; n = 6. (b) Downregulation of omega-1 transcription in S. mansoni eggs 3 days after transduction with a single shRNA-mir-containing lentivirus (shRNA-mir-557) was compared with untreated eggs (wild type (WT) control) and eggs transduced with EV lentivirus. Relative transcription of omega-1 in eggs was assessed by qPCR 3 days after transduction; transcription was normalized against the geometric mean of Ct values for three reference genes (psdm4, ef-1-a and cox-1). Bars show mean ± s.e.m.-normalized transcription for untreated control eggs or omega-1-knockdown samples relative to the EV control eggs for three independent experiments. (c) Transcription of omega-1, ipse or kappa-5 was assessed by qPCR 3 or 10 days following transduction. Bars show the mean ± s.e.m.-normalized transcription for biological replicates (n = 3) of shRNA-mir-treated eggs of the same experiment, relative to the EV control.

using pol II-driven shRNA-mir expression cassettes (as shown here), as pol II promoter activity can be constrained by regulatory elements or tissue-specific endogenous miRNAs.

Using the present lentiviral transduction system, we demonstrated that omega-1 knockdown in S. mansoni eggs led to a significant decrease in granuloma size in mice. The increased infiltration of DCs, T-helper and B cells, and interstitial macrophages seen in the lungs of EV control mice was consistent with that expected in egg-induced disease and was clearly abrogated by omega-1 knockdown. Interestingly, although a decrease in B and T cells was detected in omega-1 knockdown groups, the cytokine profile remained unaltered following re-stimulation with SEA. This finding might relate to the fact that cell numbers in the re-stimulation assay were normalized against leukocyte numbers and not against total cell numbers in lung cell suspensions. Nonetheless, considering the largely diminished infiltration of effector cells in mice injected with omega-1 knockdown eggs, it is likely to be that the inflammatory milieu in the lungs is reduced compared with WT and EV control groups. However, the diminished infiltration of B and T cells, as well as macrophages, raises questions as to which cells are responsible for the cytokine secretion and how these cells are activated. Candidate innate immune cells are eosinophils (interleukin (IL)-4 and IL-13), mast cells (IL-1, IL-6 and IL-13) and basophils (IL-4 and IL-13). Moreover, natural killer T cells have been reported as a source of IL-4 and IL-13 (ref. 30). The data from the present and other studies of granuloma formation indicate that, although an anti-inflammatory environment can be established in the absence of CD4+ T cells, Th2 effector cells are required to boost the IL-4 and IL-13 dominant cytokine milieu to induce granuloma formation.

The present study also suggests that the cytotoxicity of omega-1 (ref. 34) is a critical factor in the initiation of granuloma formation, leading to tissue destruction and activation of an innate immune response, which is maintained and amplified by Th2 cells. Indeed, Loke et al. showed that alternative activation is an innate response to injury that requires CD4+ T cells to be sustained during a chronic infection. Therefore, the change in macrophage numbers on knockdown suggests a new role for omega-1 relating to the recruitment of macrophages into tissues and involvement in fibrosis/granuloma formation. Alternatively activated macrophages (AAMs) play a critical role in the prevention of severe disease development during S. mansoni infection, as they are essential for both wound-healing processes and the suppression of severe fibrosis. Furthermore, macrophage-derived transforming growth factor-β1 has been linked to the activation of collagen production associated with healing and fibrosis. Moreover, AAMs upregulate expression of the mannose receptor, the receptor responsible for the uptake of omega-1 by DCs and its modulatory effect on DCs. Thus, it would be informative to investigate whether omega-1 can modulate AAMs to promote their immunosuppressive effect(s) after binding to the mannose receptor.

Despite the diminished cell infiltration into lung tissues, granuloma formation was not entirely abolished in the omega-1 knockdown group. A possible explanation is an incomplete suppression of omega-1 expression in eggs linked to the formation of small granulomata. Interestingly, IPSE has also been shown to be cytotoxic and might be responsible for the...
**Figure 3 | Pathological changes in the lungs of BALB/c mice injected with omega-1, ipse or kappa-5 knockdown (kd) eggs of *S. mansoni*.** Test mice were injected into the lateral tail vein with 1,000 eggs previously transduced with lentiviruses containing gene-specific shRNAmir stems (omega-1, n = 5; ipse, n = 6; kappa-5; n = 7). Control groups of mice received PBS (naive; n = 6), 1,000 untreated wild type eggs (WT; n = 7) or 1,000 eggs previously transduced with a lentivirus lacking the shRNAmir stem (that is, empty-vector (EV); n = 6). All mice were euthanized 15 days after injection. **(a)** Total leukocyte numbers in cell suspensions from the right lung; cell populations: eosinophil granulocytes (EG), neutrophil granulocytes (NG), dendritic cells (DC), T-helper cells (Th), B cells (BC), interstitial macrophages (IM) or alveolar macrophages (AM). The percentage of leukocyte populations was established by flow cytometry (see Supplementary Fig. 2 for cell gating) and calculated as the total cell number of live cells isolated from the right lung from individual mice. Box plot representing the median and upper/lower quartile. Whiskers indicate the highest or lowest value. Kruskal–Wallis and Dunns post-hoc tests were used to establish statistical significance compared with the EV control: **P < 0.01.** **(b)** Effect of omega-1, ipse or kappa-5 knockdown of *S. mansoni* eggs on total serum IgE levels in mice 15 days following injection of eggs. **(c)** Effect of egg knockdown (kd) on granuloma formation in the left lung. Granuloma sizes were determined as the ratio of the granuloma area to the egg area. Scatter plot with mean ± s.e.m. of the data pool representing all granulomata from all lungs from each experimental group (WT: n = 48; EV: n = 82; omega-1 kd: n = 77; ipse kd: n = 74; kappa-5 kd: n = 84). Kruskal–Wallis and Dunns post-hoc tests were used to establish statistical significance compared with the EV control: *P < 0.05, **P < 0.01, ***P < 0.001; not significant (ns). Numbers of mice: n = 4 (WT); n = 5 (EV, omega-1 kd); n = 6 (ipse kd) and n = 7 (kappa-5 kd). **(d)** Mean granuloma size in left lung sections from mice examined microscopically (original magnification: × 200), following staining with Masson–Goldner Trichrome. Scale bars, 100 μM. Arrows indicate *S. mansoni* eggs.
induction of smaller granulomata in the omega-1 knockdown group. However, as IPSE is the most abundant egg-secreted protein\(^43,44\), it is conceivable that, in the case of an assumed IPSE-induced cytotoxicity, the impact on granuloma formation might have been more severe. A possible scenario is that, in the absence of omega-1, IPSE can activate basophils\(^45\) to secrete IL-4 and IL-6 at levels that are not excessive, because they are not amplified by Th2 effector cells. Therefore, IPSE might contribute to wound-healing processes to counter-regulate minor tissue damage caused by translocating eggs. The moderate knockdown of ipse transcripts and the observed phenotype might reflect the presence of multiple copies of the ipse gene in the genome of S. mansoni.

Interestingly, we found that large granulomata induced by kappa-5 knockdown eggs contained more collagen compared with the WT and EV control groups (cf. Fig. 3d). Although the collagen content in tissues was not quantified, the data suggest that kappa-5 has a modulatory, anti-fibrotic effect and is involved in the regulation of fibrosis. Owing to its molecular weight, kappa-5 is retained within schistosome eggs\(^46\) and might only become accessible to the immune system when eggs disintegrate. However, in the present study, both the eggshell and the miracidium appeared to be intact, contradicting this theory. As kappa-5 can bind to C-type lectin receptors\(^37\), it would be interesting to assess whether this protein has a modulatory effect on macrophages that regulates collagen synthesis. Although kappa-5 is the only molecule presently known to induce IgE responses in people with schistosomiasis\(^36\), kappa-5 knockdown did not lead to a reduction in total serum-IgE levels in mice in the present study (Fig. 3b). However, an effect on kappa-5-specific serum IgE levels cannot be excluded, such that further work is warranted to address this aspect.

In conclusion, using a functional genomic–phenomic approach, the present study provides evidence that a key mechanism underlying S. mansoni egg-induced pathological changes is tissue destruction caused by omega-1, facilitated by its immunomodulatory capacity. This study paves the way for future investigations of schistosomiasis using complementary post-genomic tools. Clearly, the lentivirus transduction system applied here, for the first time to any parasite, has major potential to be used broadly for functional genomic–phenomic investigations of socioeconomically important eukaryotic parasites.

**Methods**

**Maintenance of S. mansoni.** All experiments were approved by the Animal Ethics Committee of the University of Melbourne and performed in quarantine-approved facilities. The NMRI strain of S. mansoni was maintained in Biomphalaria glabrata (NMRI strain, NIH-NIAID Schistosomiasis Resource Center, USA; http://www.schisto-resource.org) and female, specific pathogen-free BALB/c mice (7–8 weeks of age)\(^48\).

**Lentivirus vector and production.** The lentivirus vector pGIPZ\(_{mCherry}\) was based on pGIPZ (Open Biosystems) in which shRNA constructs are expressed as human microRNA-30 (miR30) primary transcripts (shRNAmir). The CMV promoter was inserted into the pGIPZ CMVmCherry reporter plasmid and then introduced into E. coli (Stratagene). Lentivirus vector pGIPZ\(_{mCherry}\) (via XhoI and SpeI) was inserted into the packaging plasmid pCMV-mCherry (via XbaI and SpeI), which was inserted into the helper plasmid pCMVdelta8 (via Pmel and EcoRI). To this, the mCherry reporter protein sequence from pFPVmCherry\(^49\) was inserted into the pGIPZ\(_{mCherry}\) construct (via XbaI and SpeI) to yield pGIPZ\(_{CMVmCherry}\). The IRES/puromycin resistance region was excised from the plasmid (via NotI and MluI) and substituted with the miR30 flanks produced by conventional PCR. The EcoRI restriction site encoded in the miR30 flank sequence was deleted by PCR. To do this, the 3’-miR30 region was amplified without the EcoRI sequence, and the original 3’-miR30 region was substituted with the AECori ampiclon (via BamHI and MluI restriction sites). All plasmid constructs were maintained in Escherichia coli.
coli (strain Sbi5; Invitrogen). Virus-cassette sequences were verified by automated, capillary (Sanger) sequencing. Virus was produced in TLA-HEK293 cells using the Trans-Lent shRNA Packaging Kit (Open Biosystems) with calcium phosphate. Virus titres were estimated by quantitative PCR using primers F (5′-TGGACAGGGGCCTGCGCTT-3′) and R (5′-TGGCTGTTAGTGAAGGCGGCA-3′), following the removal of residual plasmid DNA by treatment with DNase I (NEB). The functional titre of the calibrator (that is, EV control virus) was determined by calculating fluorescent cell colonies according to the manufacturer’s instructions (Open Biosystems). Resultant regression fit equations were used to estimate unknown titres; generally, virus titres of 1–5 × 10^6 transducing units per ml were used.

Cloning and validation of shRNAmir constructs. Novel, mature, artificial miRNA sequences encoded in the antisense strand of the shRNAmir stem and targeting coding regions of relevant messenger RNAs were inferred using the algorithm DSI34. Each shRNAmir-557 was designed according to the binding site in the mature sequence of the respective mRNA target. For each target gene, various shRNAmir sequences were initially selected and individually cloned into the lentiviral shRNAmir expression vector27,28 via Xhol and BamHI sites. The sequence of each cloned insert was verified by Sanger sequencing before testing in COS7 cells.

The full-length cDNA sequences of omega-1, pse and kappa-5, spanning the signal peptide-coding region and 3′-untranslated region (3′-UTR), were individually PCR-amplified from cdNA and introduced into the mammalian expression vector pcDNA3.1 myc-His (Invitrogen) via HmdIII and Xhol. Insertions were each verified by sequencing. Protein-expressing cell lines were established by transfecting COS7 cells with linearized pcDNA3.1 plasmids using the Optifect reagent (Invitrogen) and selected using G418 (Invitrogen) over 7–10 days. Clones were analysed for transgene transcription by conventional PCR from cdNA. Next, the functionality of individual shRNAmir was assessed by transfection of the shRNAmir-557 and shRNAmir-638 (5′-CAGUCCUUGACGUGACGUGGA-3′ and shRNAmir-658) based on homology to annotated 3′-UTRs of 3′-UTR sequences sharing complementarity to the mature artificial miRNA ‘seed’ region (nt 2–8) were predicted to be potential off-target sites34,46. The sequences for the mature artificial miRNAs (5′-3′) used here were as follows: UGGAUUGAUUGAUUGUCUG (shRNAmir-511), UAGCAACCUACUGUACAUG (shRNAmir-537), UAGGAGUGAUGUGUGUGUGCUG (shRNAmir-195), UAGGAAACAAUCUGUACUUCU (shRNAmir-384), UUUAGUGUUGGUAUUUCAUC (shRNAmir-616) and UUGAUCUAGAUAGAUCUG (shRNAmir-638).

Transduction of S. mansoni eggs. Following their isolation from mouse livers48, schistosome eggs were washed extensively in sterile PBS; individual batches of 3,000 eggs were then exposed to lentivirus particles at a multiplicity of infection of 10, or to untreated (WT) control in 500 μl of serum-free DME MEM (Gibco) and 8 μg ml⁻¹ of polybrene (Sigma). Virus without shRNAmir stem (that is, EV) or viruses each containing the gene-specific shRNAmir stems omega-1, pseRNAmir-557 or shRNAmir-384; and kappa-5 shRNAmir-616 or shRNAmir-658 were used. After an incubation for 24 h at 37°C in 5% CO₂, individual egg batches were washed extensively in PBS (37°C) to remove virus particles and polybrene. Then, individual egg batches (n = 3,000) were cultured for (2–9) 2–3 days in DME with 10% FCS and 2 mM L-glutamine and egg genomic DNA isolated with TRIzol (Invitrogen). Subsequently, the presence of viral DNA was verified by conventional PCR using specific primer sets and Subtractive blot analysis46 of genomic DNA of S. mansoni. The maturity and viability of eggs were assessed daily by microscopy examination. Washing of the egg batches was induced 10 days after virus exposure and monitored microscopically.

Assessing gene knockdown following transduction. The knockdown of target gene transcription in cultured S. mansoni eggs was assessed by qPCR. To do this, total RNA was isolated from individual batches of 3,000 eggs using TRIzol (Invitrogen) and residual DNA removed by DNase I treatment; cdNA was synthesized from 250 ng of RNA by reverse transcription using random primers and MMLV reverse transcriptase (Bioline). Following the calculation of PCR efficiencies58, which ranged between 1.9 and 2.1, the relative transcription (R) of individual target genes was related to EV control samples (that is, mean Ct value), and transcription was normalized against those of cos-1, ef-1a and psdm-4 after 3 days of in vitro culture; or act-1b59 after 10 days of in vitro culture, according to the efficient comparative quantification model for multiple samples60. The primers used were: for the cos-1, ef-1a and psdm-4 were using the geNorm tool of the qBASEPLUS software (Biogazelle), employing established principles61.

Mouse experimentation and pathological changes. Following the verification of gene knockdown, virus-treated and untreated egg batches were introduced into mice in a sterile condition to assess survival and pathological changes. To do this, 1,000 eggs in 100 μl of sterile PBS were injected into the lateral tail vein of female BALB/c mice (7–8 weeks of age)55. Individual mice in treatment groups were each injected with PBS (naive), untreated eggs (WT) or with eggs previously transduced with a lentivirus lacking the shRNAmir stem (that is, EV). Mice were euthanized 15 days after injection; immediately, individual lungs were perfused with 10 ml of sterile PBS (4°C) via the heart, after which they were extracted from the thoracic cavity, and connective tissue and mediastinal lymph nodes trimmed off. Then, four investigatory components were conducted.

First, from individual right-lung lobes, single-cell suspensions were prepared52. Residual erythrocytes were lysed using ACK solution (Gibco) and cells resuspended in PBS containing 1% w/v of BSA. Total leukocyte counts were established by flow cytometry (FACS Calibur, BD Bioscience, USA) using Countbright absolute counting beads (Invitrogen); dead cells were excluded using 7- amino-actinomycin D (Sigma). Lung cell suspensions were incubated in FACS-staining buffer (PBS containing 1% BSA and 5 μM of EDTA) with LEAF-purified anti-mouse CD16/32 (clone 93; BioLegend) and the following antibodies (BioLegend): anti-CD11c (clone N418), -CD3e (clone 145-2C11); R-phcoerthrin-conjugated anti-CD4 (clone RM4-5, Invitrogen), -F4/80 (clone BM8), -Siglec-F (clone E54-2440, BD Bioscience); fluorescein isothiocyanate-conjugated anti-b220 (clone RA3-682); -Ly-6G/Ly-6C (RB6-8C5). All antibodies were used at a 1:2 dilution, except anti-mouse F4/80 (1:100). Pooled cells from all mice of individual groups were also stained with antibody mixes with antibody mixes for the respective isotypes. The LIVE/DEAD Fixable Red stain (Invitrogen) was used to exclude dead cells. Flow cytometric analyses were carried out with a FACS Calibur and CellQuest software (BD) and analysed with FlowJo (Tree Cell; Gating; see Supplementary Fig. 2). Second, left-lung lobes were subjected to histopathological examination of all individual lobes scored in 10 normal areas (0.2 mm (x) × 0.7 mm (y) ) or embedded in Tissue-Tek OCT compound (Sakura Finetek), serially sectioned (5–8 μm), stained with haematoxylin–eosin or Masson’s Trichrome stain and then mounted in DPX (Sigma). Samples were examined for granuloma containing a centrally sectioned oval area with a BX60 microscope (Olympus) equipped with a SPOT camera (Diagnostic Instruments) and Image-Pro software (MediaCybernetics).

Subsequently, for individual granuloma, the granuloma area and the egg area were measured by computerized morphometry and the ratio between the two areas calculated. Third, total IgG levels in sera from individual mice were measured using the Ready-Set-Go mouse IgG ELISA kit (eBioscience), according to the manufacturer’s instructions, using a Synergy H1 Hybrid Reader (BioTek). Fourth, lung cell suspensions were restimulated with SEA (20 μg ml⁻¹) for 72 h, and culture supernatants assayed using the Mouse Th1/Th2/Th17/Th22 13-plex kit FlowCytomix (eBioscience).

Statistical analyses. Treatment groups were analysed for significant differences using the Kruskal–Wallis one-way analysis of variance (P<0.05) and Dunn post-hoc tests63 in relation to the EV control group (n = 5–7) using Prism software (Graph-Pad Software Inc.).

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