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Basic

Short Communication

Immunization with excretory-secretory molecules of intestinal nematodes induces antigen-specific protective memory Th2 cell responses

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Parasitic nematodes infect more than 1 billion people in the global south. The development of effective antihelminthic vaccines is a crucial tool for their future elimination. Protective immune responses to nematodes depend on Gata3$^+$ Th2 cells, which can also be induced by nematode-released products. Whether these nematode products induce antigen-specific long-lived memory T cells and thereby confer protection against a challenge infection is not known yet. Hence, we set out to characterize the formation of memory Th2 cells induced by immunization with *Heligmosomoides polygyrus* excretory-secretory (HES) products, infection-induced versus immunization-induced recall responses to a challenge infection, and whether HES-induced memory T cells show protective properties following adoptive transfer. Our results show that 8 weeks postimmunization, HES induces long-lived functional memory Th2 cells at the site of immunization in the peritoneal cavity. Following a *H. polygyrus* challenge infection, HES-immunized mice display MHC-II-dependent antigen-specific Th2 cytokine responses in the gut-draining lymph nodes, comparable to those induced by a prior natural infection. Moreover, adoptive transfer of sorted memory CD4$^+$ T cells from HES-immunized donors reduces female worm fecundity following a challenge *H. polygyrus* infection in recipient mice, highlighting a protective role for immunization-induced memory T cells.

Keywords: memory Th2 cells · nematode · *H. polygyrus* · HES · immunization

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Intestinal soil-transmitted helminths (STHs) affect nearly one quarter of the world population and greatly contribute to chronic disease burdens in endemic populations [1, 2]. Although anthelminthic drug therapy is key to interrupting transmission, alone it is insufficient to effectively eliminate STHs, evidenced by the occurrence of high rates of reinfections in communities in endemic regions [3–5]. The development of effective anti-helminth vaccines is therefore vital for the future elimination of parasitic helminths [6]. Moreover, due to the common occurrence of co-infections with two or more parasitic helminths, the development and availability of multivalent cross-protective vaccines is a particularly desirable immunization approach [7, 8].

The murine small intestinal nematode *Heligmosomoides polygyrus* represents a natural model for characterizing both host immune responses to natural infection and the efficacy of experimental vaccine candidates. We and others have previously shown that mice develop strong protective responses against

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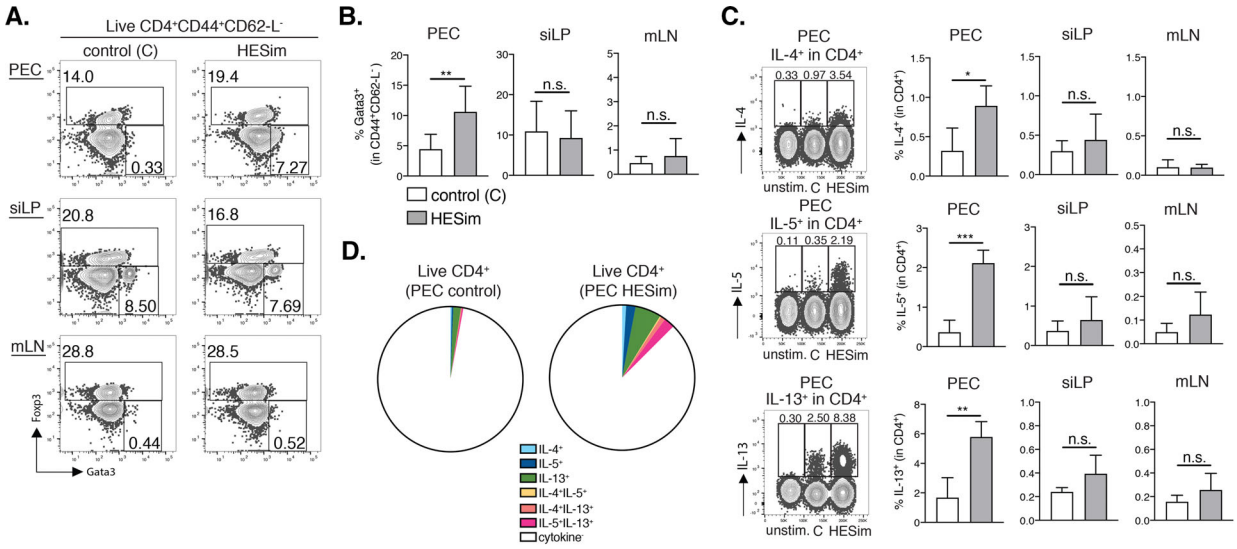

Figure 1. HES immunization induces localized long-lived functional memory Th2 cells. (a) Example FACS plots showing Gata3 and Foxp3 expression in live $CD4^+CD44^+CD62\text{-}L^-$ memory T cells in the PEC, siLP, and mLN of C57BL/6 mock-immunized controls (C) and HES-immunized mice (HESim). (b) Frequencies of $Gata3^+$ memory Th2 cells. (c) Example FACS plots and frequencies of IL-4^+, IL-5^+, and IL-13^+CD4^+ T cells. (d) Pie chart illustrating the proportions of cytokine$^+$ $CD4^+$ T cells in the PEC of C and HESim. The data are pooled from two independent experiments, with a total $n = 6$ for control mice and $n = 10$ for HESim and represent mean ± SD. Statistical analysis in (b) and (c) was done using an unpaired *t*-test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, n.s.: not significant.

reinfections with parasitic nematodes, dependent on memory Th2 cells, IL-4, and eosinophil responses [9–15]. Prior *H. polygyrus* infection was recently demonstrated to also enhance $CD4^+$ T-cell-associated cross-protection against a challenge infection with the tissue-migratory nematode *Nippostrongylus brasiliensis* [16]. In the context of immunization-induced protection, several studies have used nematode-derived products like *H. polygyrus* excretory-secretory (HES) molecules, demonstrating that HES-immunized mice display strong protective immune responses against a challenge nematode infection [17, 18]. Transfer of HES-specific monoclonal antibodies did not induce protection against a challenge *H. polygyrus* infection, indicating that HES-induced humoral immunity alone is insufficient and that immunization-induced host protection also requires cellular immunity [17]. Here, we assessed whether HES immunization induces functional long-lived memory Th2 cells with protective properties against a challenge *H. polygyrus* infection, as well as whether HES-immunized mice develop cross-reactive immune responses against the unrelated nematode *Ascaris suum*, as an experimental measure of the feasibility of developing multivalent anti-helminthic vaccines.

Results and discussion

HES immunization induces a localized memory Th2 cell population

To assess the ability of HES to induce functional memory T cells, we quantified the frequencies of $Gata3^+CD4^+CD44^+CD62\text{-}L^-$ memory Th2 cells in the peritoneal exudate cells (PEC), small intestinal lamina propria (siLP), and mesenteric LNs (mLN) of HES-immunized mice and alum-immunized controls (C) (Fig. 1a; Supporting information Fig. S1a). Eight weeks postimmunization, HES induced a significant increase in memory Th2 cell frequencies, but not immunosuppressive regulatory T cells (Treg), at the site of immunization in the PEC, while in the siLP and mLN no significant changes were observed between C and HES-immunized mice (Fig. 1b; Supporting information Fig. S1a and b). HES-immunized mice also harbored significantly elevated frequencies of IL-4^+, IL-5^+, and IL-13^+CD4^+ T cells in the PEC, compared with mock-immunized controls (Fig. 1c). Moreover, HES immunization induced the expansion of polyfunctional peritoneal T cells, in particular IL-5^+IL-13^+ co-producing cells (Fig. 1d). Our data therefore indicate for the first time that HES immunization induces cytokine-competent long-lived memory Th2 cells at the site of immunization.

HES-induced parasite-specific Th2 responses are comparable to those induced by a natural infection

Next, we compared recall Th2 responses to a challenge *H. polygyrus* infection following either infection-induced or HES-induced protection (Fig. 2a). Quantifying adult worm burdens and female worm fecundity in mock-immunized and challenged (1° Hp), cured and challenged (2° Hp), and HES-immunized and challenged mice (HES-Hp), we confirmed that immunized mice exhibit significant protection against reinfection, like that observed in cured and challenged mice (Fig. 2b), in line with previous studies [18, 19]. Cured mice displayed an overall trend for higher frequencies of intestinal Th2 cells and significantly elevated Th2 activation based on CD69 expression, compared with

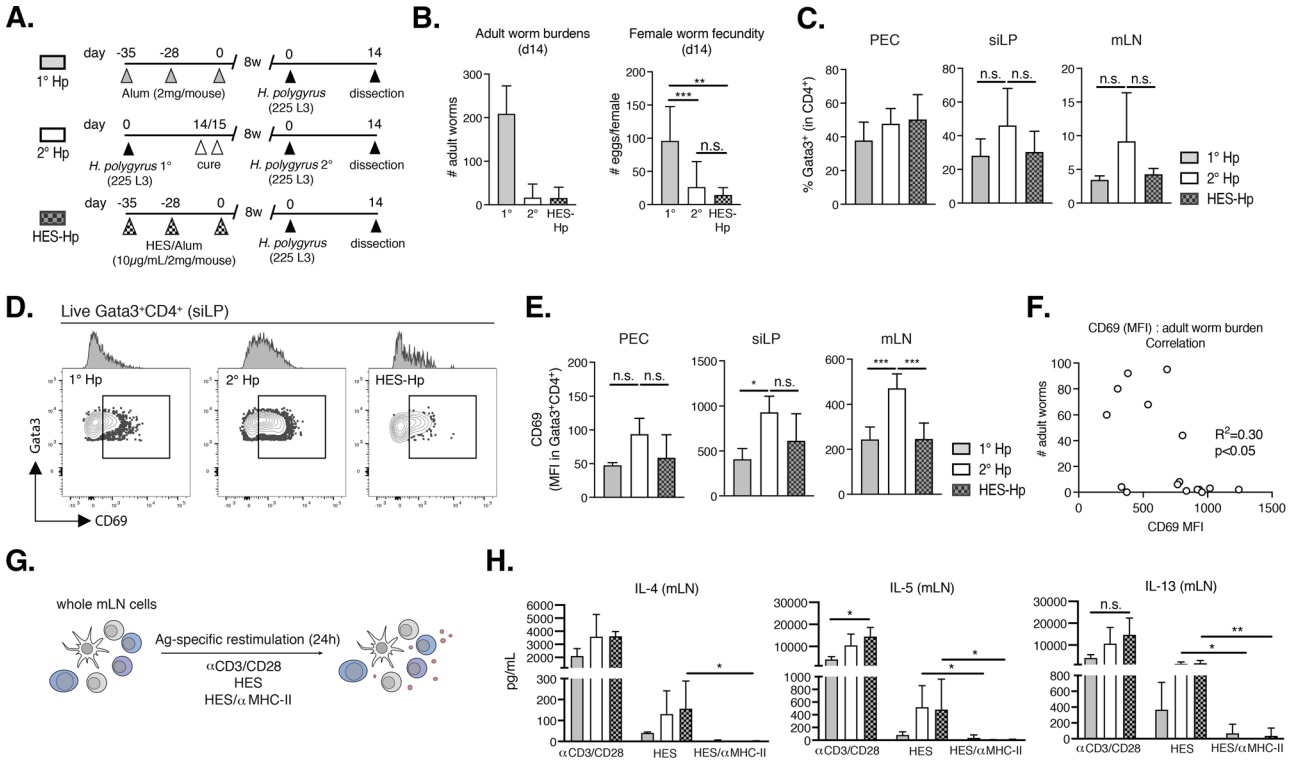

Figure 2. HES-immunized mice harbor strong antigen-specific Th2 recall responses against a challenge *H. polygyrus* infection. (a) Experimental set-up, using C57BL/6 mice. (b) Small intestinal adult worm burdens and female worm fecundity. (c) Frequencies of Gata3$^+$CD4$^+$ Th2 cells in the PEC, siLP, and mLN, shown within total CD4$^+$ T cells, rather than CD44$^+$CD62-L$^-$. The presence of an acute nematode infection involves both the induction of de novo effector Th2 cells and the reactivation of memory Th2 cells into an effector state, making a distinction between CD44$^+$CD62-L$^-$ memory cells and effector cells obsolete. (d) Example FACS plots with adjunct histograms of CD69 expression in Th2 cells from the siLP. (e) Median fluorescence intensity (MFI) of CD69 expression in Th2 cells from the PEC, siLP, and mLN. (f) Correlation analysis of CD69 expression on siLP Th2 cells and adult worm burdens. (g) Experimental set-up of in vitro restimulated mLN cells. (h) Levels of IL-4, IL-5, and IL-13 protein in the supernatants of in vitro restimulated mLN cells. The data are pooled from two independent experiments, with a total $n = 6$ for 1° Hp mice, $n = 10$ for 2° Hp mice, and $n = 7$ for HES-Hp mice and represent mean ± SD. Statistical analysis in (b), (c), (e), and (h) was done using one-way ANOVA combined with Tukey's multiple comparisons test. Statistical analysis in (f) was done using the Spearman correlation test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, n.s.: not significant.

HES-immunized mice (Fig. 2c-e), indicative of a mild differential effect of a prior natural infection versus immunization on host Th2 recall responses. Nevertheless, correlation analysis of CD69 expression in siLP Th2 cells against adult worm burdens revealed a significant negative correlation, highlighting that cell activation, rather than overall frequencies of Th2 cells, influences host control of *H. polygyrus* (Fig. 2f).

We also investigated the effects of HES immunization on antigen-specific Th2 responses. For this, whole mLN cells from 1° Hp, 2° Hp, and HES-Hp mice were restimulated in vitro for 24 h with αCD3/CD28 antibodies to quantify the overall polyclonal cytokine response. HES and anti-MHC-II blocking antibodies (αMHC-II) were also added to quantify the adaptive recall response to parasite antigens (Fig. 2g). We found that HES induces strong parasite-specific IL-4, IL-5, and IL-13 recall responses to *H. polygyrus*, comparable to those induced in 2° Hp mice. Moreover, the parasite-specific cytokine release of cells from both 2° Hp and HES-Hp mice was abrogated upon blocking MHC-II signaling, indicating that HES immunization is a potent inducer of parasite-specific Th2 responses, comparable to those induced by a prior natural infection (Fig. 2h). Our findings therefore offer new insights into the ability of HES to induce long-lived memory Th2 responses and strong parasite-specific Th2 recall responses against *H. polygyrus*.

HES-induced memory T cells show protective functions upon adoptive transfer

Having demonstrated that HES immunization induces long-lived cytokine-competent memory Th2 cells and that immunization-induced recall responses are comparable to those induced by a prior natural infection, next we tested whether HES-induced memory T cells contribute to protection against a challenge nematode infection. For this, donor mice were immunized with HES. Eight weeks post-immunization, naïve CD4$^+$CD44$^-$CD62-L$^+$ and memory CD4$^+$CD44$^+$CD62-L$^-$ T cells were sorted from the peritoneal cavity of immunized donors and were adoptively transferred via an intraperitoneal injection to naïve recipients (Fig. 3a). One day posttransfer, the recipient mice

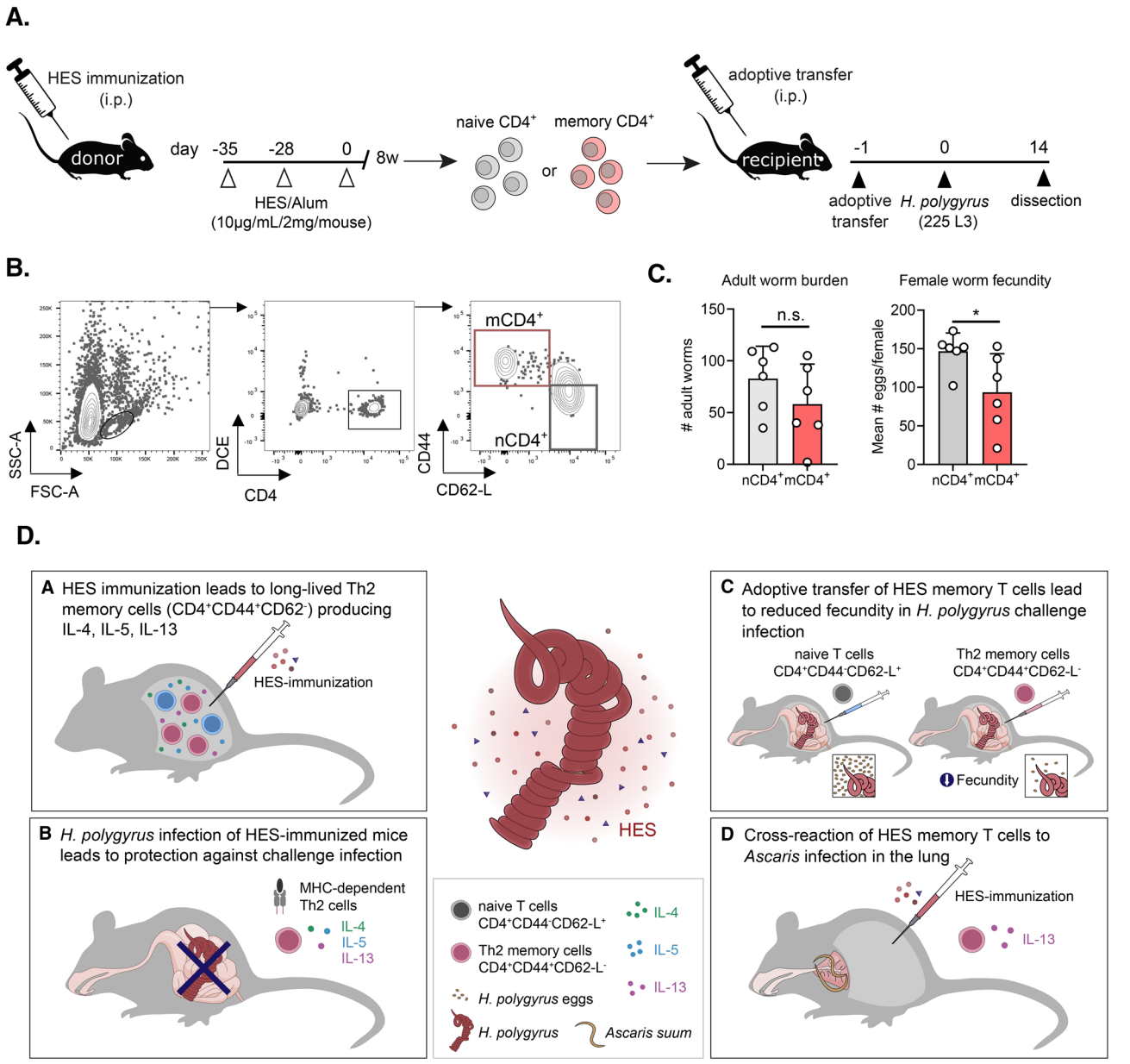

Figure 3. HES-induced memory T cells show protective properties against a challenge nematode infection. (a) Experimental set-up, using C57BL/6 mice. Briefly, donor mice were immunized with HES and 8 weeks after the final immunization, and naïve $CD4^+CD44^-CD62\text{-}L^+$ ($nCD4^+$) and memory $CD4^+CD44^+CD62\text{-}L^-$ T cells ($mCD4^+$) were sorted from peritoneal exudate cells (PEC). Sorted T cells were adoptively transferred via intraperitoneal injection to naïve recipients. One day postadoptive transfer, recipient mice were challenged with a *H. polygyrus* infection and were dissected 14 days postinfection. (b) Example sorting strategy plots. (c) Adult worm burdens and female worm fecundity (mean numbers of eggs excreted, calculated using egg excretion counts of eight female worms per mouse) in $nCD4^+$ and $mCD4^+$ recipient mice at 14 days post-*H. polygyrus* challenge infection. (d) Graphical summary. The data are pooled from two independent experiments, with a total $n = 6$ for $nCD4^+$ and $mCD4^+$ groups each and represent mean ± SD. Statistical analysis in (c) was done using an unpaired *t*-test. $^*p < 0.05$, n.s.: not significant.

were challenged with a *H. polygyrus* infection and were dissected 14 days later (Fig. 3a and b). Interestingly, we found that while memory T-cell recipients ($mCD4^+$ group) showed only a trend for lower adult worm burdens, female worm fecundity was significantly decreased in $mCD4^+$ compared with the naïve T-cell ($nCD4^+$) recipients, despite overall similar $Gata3^+$ Th2 cell responses in the different tissues (Fig. 3c, Supporting information Fig. S2). Previously, we were able to show that adoptive transfer of sorted total $CD4^+$ T cells from the peritoneum of *H. polygyrus*-cured mice induces similar reductions in worm fecundity in nematode-challenged recipients, without notable shifts in Th2 responses [14]. In line with this, our current findings therefore extend our previous work and confirm that HES-induced memory T cells directly contribute to protection against a challenge *H. polygyrus* infection (Fig. 3d).

Nematode-induced memory Th2 cells have previously been shown to confer protection to reinfection in an IL-4 and eosinophil-dependent manner [10, 12]. Here, we only observed a mild trend for elevated IL-4$^+$ T cell frequencies in the siLP of mCD4$^+$ mice following *H. polygyrus* challenge, while eosinophil responses remained beyond the scope of the current study. Nevertheless, considering the reductions in female worm fecundity, it is reasonable to assume that infection-induced and HES immunization-induced memory T cells contribute to host recall responses via additional downstream Th2 mechanisms such as eosinophil or macrophage induction in response to a challenge nematode infection.

Prior HES immunization induces limited cross-reactive Th2 responses against unrelated nematodes

Finally, we asked whether prior HES immunization enhances host control of a challenge infection with another tissue-migratory nematode, *Ascaris suum*. For this, mock-immunized (As) and HES-immunized (HES-As) mice were challenged with an *A. suum* infection 8 weeks post-HES immunization (Supporting information Fig. S3a). At 8 days post-*Ascaris* infection, we found comparable lung larval loads in As and HES-As mice (Supporting information Fig. S3b). Similarly, despite seeing a trend for stronger Th2 responses in HES-As mice, Gata3$^+$ Th2 cell frequencies, as well as IL-4$^+$ and IL-13$^+$ cell frequencies, were overall comparable in lung and PEC between As and HES-As groups (Supporting information Fig. S3c and d). Nevertheless, in in vitro restimulated whole lung cells from As and HES-As mice, we detected distinct cytokine responses to HES and *A. suum* larval antigens (AsAg). Most importantly, we detected enhanced AsAg-specific IL-13 release (Supporting information Fig. S3e and f). Overall, our data therefore indicate that even though HES immunization does not alter larval burdens or overall Th2 responses in *Ascaris*-infected hosts, HES-immunized individuals displayed elevated *Ascaris* antigen-specific lung IL-13 release, indicating a limited potential of HES immunization to induce cross-reactive immune cell responses against unrelated nematodes (Fig. 3d).

Concluding remarks

In the current study, we show that HES immunization induces a long-lived localized population of functional memory Th2 cells in the peritoneal cavity of HES-immunized mice. Moreover, upon a *H. polygyrus* challenge infection 8 weeks postimmunization, HES-immunized mice display long-term protection and notable MHC-II-dependent nematode-specific cytokine responses, comparable to those induced by a prior natural infection. In addition, we are able to show that adoptive transfer of HES-induced memory T cells significantly reduces female worm fecundity upon challenge nematode infection, highlighting a protective function of immunization-induced memory T cells. Finally, we show that prior HES immunization induces a cross-reactive antigen-specific IL-13 response in the lungs of *Ascaris*-infected mice (Fig. 3d). Our study, therefore, offers new insights into long-term host cellular immunity induced by helminth-released products.

Materials and methods

Mice, HES immunization, and nematode infections

Wild-type 8- to 10-week-old female BALB/c and C57BL/6 mice were purchased from Janvier Labs (Saint-Berthevin, France). The animals were maintained under specific pathogen-free conditions and were fed standard chow ad libidum. For primary and challenge infections, mice were infected with either 225 third-stage infective (L3) *H. polygyrus* larvae (C57BL/6) or with 1000 embryonated *A. suum* eggs (BALB/c) via oral gavage. For cure of primary *H. polygyrus* infections, mice were treated with pyrantel pamoate as previously described [14]. For quantification of female worm fecundity, eight female *H. polygyrus* worms per mouse were isolated from the small intestine and were plated out in a 96-well plate in individual wells containing 150 μL of cRPMI medium. The worms were then incubated at 37°C for 24 h, after which the numbers of eggs excreted by individual worms were counted using a Neubauer chamber under a light microscope. From each eight female worms per mouse, the mean numbers of eggs excreted were then calculated in order to obtain the mean female worm fecundity per mouse. HES products were collected and processed as previously described [20]. Mice were immunized with of 1 μg HES per mouse (10 μg/mL HES and 2 mg/mouse alum in 100 μL sterile PBS) intraperitoneally three times at indicated timepoints and were allowed to rest for 8 weeks after the final immunization before nematode challenge infections. Mock-immunized mice were given alum in PBS. Mice were sedated using xylavet/ursotamine administration, followed by cervical dislocation. All animal experiments were performed in accordance with the National Animal Protection Guidelines and approved by the German Animal Ethics Committee for the Protection of Animals (LAGeSo, G0176/20).

Preparation of single cell suspensions

The isolation of single cell suspensions from the PEC, siLP, mLN, and lungs was performed as previously described [14].

Adoptive transfer of naïve and memory CD4$^+$ T cells

Allocated groups of C57BL/6 donor mice were immunized as described above. Eight weeks postimmunization, the donor mice were dissected, and PEC single cell suspensions were pooled together. The pooled PEC cells were then stained using fluorescently labeled anti-CD4, CD44, and CD62-L antibodies. Naïve CD4$^+$CD44$^-$CD62-L$^+$ and memory CD4$^+$CD44$^+$CD62-L$^-$ T cells

were sorted on an Aria cell sorter (BD Biosciences, Heidelberg, Germany). Approximately 200 000 naïve or memory T cells per mouse were adoptively transferred to recipient mice via intraperitoneal injection. One day postadoptive transfer, recipient mice were then orally challenged with a *H. polygyrus* infection and were dissected 14 days postinfection.

Flow cytometry

The list of antibodies used for flow cytometry is described in Supporting information Table S1. Dead cells were excluded using eFluor780 or eF506 fixable viability dye (Thermo Fisher, Waltham, USA). For the intracellular staining of transcription factors and cytokines, cells were fixed and permeabilized using the Foxp3 Fixation/Permeabilization kit and Permeabilization buffer from eBioscience. Samples were analyzed on a Canto II flow cytometer and on an Aria cell sorter (BD Biosciences, Heidelberg, Germany). The data were analyzed using FlowJo software version 10 (Tree star Inc., Ashland, OR, USA) and adhered to the "Guidelines for the use of flow cytometry and cell sorting in immunological studies" [21].

Cell culture and in vitro antigen-specific restimulation

For the analysis of parasite-specific cytokine responses, 5×10^5 whole mLN or lung cells per well were plated out in a round-bottom 96-well cell culture plate in a final volume of 200 μL cRPMI medium (10% FCS, 100U/mL penicillin, 100 μg/mL streptomycin; PAA, Pasching, Austria). The cells were stimulated either with HES (10 μg/mL), *A. suum* larval antigen (AsAg, 10 μg/mL), anti-CD3/CD28 antibodies (1 μg/mL), or anti-MHC-II blocking antibodies (clone M5/114.15.2, 5 μg/mL). At indicated timepoints, the cell culture supernatants were collected and stored at −20°C for later analysis of cytokine release.

Cytokine detection via ELISA

The cytokines IL-4, IL-5, and IL-13 were measured in cell culture supernatants from in vitro restimulated mLN and lung cells using mouse IL-4, IL-5, and IL-13 uncoated ELISA kits following the manufacturer's instructions (ThermoFisher, MA, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 9.0.1 (La Jolla, CA, USA). Results are displayed as mean ± SD and significance is displayed as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Results were tested for normal distribution using the Shapiro–Wilk normality tests, followed by an unpaired *t*-test or one-way ANOVA combined with Tukey's multiple comparison test.

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Conflict of interest: The authors declare no commercial or financial conflict of interest.

Ethics statement: All animal experiments were performed in accordance with the National Animal Protection Guidelines and approved by the German Animal Ethics Committee for the Protection of Animals (LAGeSO, G0176/20).

Author contributions: I.A.Y. and S.H. planned and designed the study. I.A.Y. and L.E.E.V. performed the experiments, data analysis, and interpretation. I.A.Y. and L.E.E.V. wrote the manuscript, and I.A.Y., L.E.E.V., and S.H. edited the manuscript. S.H. supervised the study and provided critical review of the manuscript.

Data availability statement: The results presented here are available from the corresponding author upon reasonable request.

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Abbreviations: **HES:** *Heligmosomoides polygyrus* excretory-secretory · **mLN:** mesenteric LNs · **PEC:** peritoneal exudate cells · **STH:** soil-transmitted helminth

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