Manuscript Title: Intestinal helminth infection impairs oral and parenteral vaccine efficacy[1,2,3]

Running Title: Intestinal helminth infection impairs vaccine efficacy

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[3]**Abbreviations:** GALT, gut-associated lymphoid tissue; LP, lamina propria, MLN, mesenteric lymph node; OT-II Tg, OT-II transgenic; RAG1 KO, recombination-activating gene 1–deficient; Treg, regulatory T cell; Teff, T-effector cell ; WHO, World Health Organization

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ABSTRACT

The impact of endemic parasitic infection on vaccine efficacy is an important consideration for vaccine development and deployment. We have examined whether intestinal infection with the natural murine helminth *Heligmosomoides polygyrus bakeri* alters antigen-specific antibody and cellular immune responses to oral and parenteral vaccination in mice. We found that oral vaccination of mice with a clinically relevant, live, attenuated, recombinant *Salmonella* vaccine that expresses chicken egg ovalbumin (*Salmonella*-OVA) disrupts ovalbumin-specific regulatory T cell networks in the gut associated lymphoid tissue and promotes T-effector responses to OVA. Chronic intestinal helminth infection significantly reduced Th1-skewed antibody responses to oral vaccination with *Salmonella*-OVA. Activated, adoptively-transferred, OVA-specific $CD4^+$ T cells accumulated in draining mesenteric lymph nodes (MLN) of vaccinated mice, irrespective of their helminth-infection status. However, helminth infection increased the frequencies of adoptively-transferred OVA-specific $CD4^+$ T cells producing IL-4 and IL-10 in the MLN. Chronic intestinal helminth infection also significantly reduced Th2-skewed antibody responses to parenteral vaccination with OVA adsorbed to alum. These findings suggest helminth-induced impairment of vaccine antibody responses may be driven by the development of IL-10-secreting $CD4^+$ T regulatory cells. They also underscore the potential need to treat parasitic infection before mass vaccination campaigns in helminth-endemic areas.

INTRODUCTION

Vaccination is one of the most effective public health measures against infection (1-4). However, as the COVID-19 pandemic has highlighted, there are significant unmet needs for vaccine coverage for adults and children across the world, particularly in low- and middle-income countries where populations shoulder a significant portion of the world's infectious disease burden (3-5). The World Health Organization (WHO) and other multinational, public-private partnership organizations continue to advocate for strategies that address the availability, affordability, storage and handling, ease of administration, and safety of vaccines, with the goal of expanding vaccination coverage amongst underserved populations (4, 6, 7).

Mucosal vaccines, including oral vaccines, are potent inducers of local mucosal and systemic cellular and humoral immune responses (8-10). Recombinant oral *Salmonella* vaccines, for example, are used in veterinary medicine, especially in the context of poultry farming, to improve fowl health and ensure food safety (11, 12). Oral vaccination with live-attenuated *Salmonella* strains is also used to protect humans against typhoid (13, 14) and paratyphoid fever (15). In humans, oral *Salmonella* vaccines activate circulating B and T cells, expand the number of circulating $CD4^+$ Th1 cells, increase serum IFN-γ and TNF-α, and induce *Salmonella*-specific serum and fecal antibody responses (14). In mice, T and B cells are also critical for protective immune responses to attenuated and virulent *Salmonella* (16). $CD4^+$ Th1 cells and robust IFN-γ production (17, 18) coupled with *Salmonella*-specific IgG and IgA responses are critical for the clearance of *Salmonella* and development of protective immunity against virulent *Salmonella* strains (18, 19).

One challenge to vaccination is the impact that parasitic gastrointestinal helminth infections can have on the immune response to vaccines (20-22). Greater than 50% of the

106 world's population lives in regions where helminth infections are endemic (23). Nearly 1.5
107 billion people are chronically infected with gastrointestinal helminths (23, 24). Three hundred
108 million of these individuals also suffer from malnutrition, stunted growth, anemia, and reduced
109 protective immunity to unrelated pathogens (25, 26). We and others have shown that preexisting
110 helminth infection is a potent modulator of the immune response to orally delivered dietary
111 antigens (27-29), gastrointestinal bacterial infection (30) and parenteral vaccination (22, 31, 32).
112 Epidemiological studies, clinical trials, and animal models suggest that helminth infection has
113 powerful immunosuppressive effects on the development of allergic, autoimmune, and
114 inflammatory diseases (33-35). Helminth infection promotes immune suppression by inducing
115 regulatory cells and cytokines that modulate Th1-, Th2-, and Th17-dependent immune responses
116 (36, 37) in part through interactions with endogenous microbiota (38, 39). Since the regions
117 where helminth infection is endemic overlap significantly with the regions of the world targeted
118 by global health organizations for improved vaccine coverage (1, 4, 40), the impact of helminth
119 infections on vaccine-induced protective immunity must be considered in vaccine design and
120 deployment.

121 We used a live attenuated oral *Salmonella* vaccine strain expressing chicken egg
122 ovalbumin (OVA) (41) to examine the impact of chronic intestinal helminth infection with the
123 natural mouse parasite *Heligmosomoides polygyrus bakeri* **(***H. polygyrus bakeri***)** on vaccine
124 antigen-specific cellular and antibody responses. We found that a live attenuated oral
125 *Salmonella*-OVA vaccine disrupts OVA-specific regulatory T cell expansion, promoting OVA-
126 specific T-effector responses. Chronic intestinal helminth infection significantly reduced Th1-
127 skewed antibody responses to oral vaccination with *Salmonella*-OVA even though activated
128 OVA-specific $CD4^+$ T cells accumulated in draining mesenteric lymph nodes (MLNs) of

helminth-free and helminth-infected mice. Helminth infection also increased the frequencies of adoptively-transferred, OVA-specific $CD4^+$ T cells producing IL-4 and IL-10 in the draining MLN. This suggests that IL-10-secreting $CD4^+$ T regulatory cells may reduce vaccine-induced antibody responses in helminth-infected mice and highlights the potential need to eliminate immunosuppressive intestinal parasites prior to vaccination in regions where helminth infection is endemic.

MATERIALS AND METHODS

Mice. To evaluate peripheral conversion of OVA-specific $CD4^+$ T cells to $Foxp3^+$ Treg cells, $Ly5.1^+$ RAG-1 replete B6.SJL mice and C57BL/6 OT-II transgenic (Tg) RAG-1 KO $Ly5.2^+$ mice were purchased from Taconic Farms. Foxp3 eGFP reporter mice ($Foxp3^{eGFP}$) were originally obtained from M. Oukka (Brigham and Women's Hospital, Cambridge, MA (42)). OT-II Tg RAG-1 KO $Ly5.2^+$ $Foxp3^{eGFP}$ mice were generated by crossing the F1 progeny of C57BL/6 OT-II Tg RAG-1 KO $Ly5.2^+$ x Foxp3 ^{eGFP} breeders. These mice were maintained at an American Association for the Accreditation of Laboratory Animal Care–accredited animal facility at the National Institute for Allergy and Infectious Diseases (NIAID) and housed following procedures outlined in the Guide for the Care and Use of Laboratory Animals under an animal study proposal approved by the NIAID Animal Care and Use Committee.

For the helminth infection and oral and intramuscular vaccination experiments conducted at Massachusetts General Hospital (MGH), six- to eight-week-old male and female C57BL/6 J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). OT-II (Thy1.1) mice on the C57BL/6 background, transgenic for the TCR recognizing OVA peptide 323-339 were provided by A. Luster (Massachusetts General Hospital (MGH), Charlestown, MA). Mice were fed autoclaved food and water and maintained in a specific-pathogen-free facility at MGH. All experiments were conducted after approval and according to regulations of the Subcommittee on Research Animal Care at MGH.

For helminth infection and intramuscular vaccination experiments conducted at University of North Carolina at Chapel Hill (UNC), eight to sixteen-week-old male and female C57BL/6 J mice were also purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were fed

158 autoclaved food and water and maintained in a specific-pathogen-free facility at the UNC. All
159 mouse experimental procedures were approved by the UNC Institutional Animal Care and Use
160 Committee.

161 ***In vivo* cell transfer and dietary oral antigen administration.** T lymphocytes were extracted
162 from the peripheral LNs (excluding the spleen) of OT-II Tg RAG-1 KO Foxp3^{eGFP} mice (Ly5.2^{+})
163 and adoptively transferred into B6.SJL recipient mice (Ly5.1^{+}). Each mouse received 10^6 cells.
164 Recipient mice were split into two groups. Select groups received a 1.5% OVA solution in
165 drinking water replaced every 48 h (grade V; Sigma-Aldrich) for five consecutive days. The
166 other groups received normal drinking water. On day 6, mesenteric lymph nodes (MLNs –
167 pooled portal, duodenum, jejunum, and ileum LNs as previously described (43)) and intestinal
168 lamina propria (LP) were collected from B6.SJL hosts, and Foxp3-eGFP expression assessed in
169 transferred cells. LN and LP single-cell suspensions were prepared as previously described (44).

170 ***S. typhimurium* vaccine strains and oral immunization.** The recombinant attenuated vaccine
171 strain *Salmonella typhimurium* SL3261 (*aroA,* (45)) carrying either the plasmid pnirOVA
172 (*Salmonella*-OVA) or pnirBEM (*Salmonella*-BEM) were grown overnight shaking at 37ºC in
173 Luria Bertani (LB) broth supplemented with 100μg/ml of ampicillin (Ap) as previously
174 described (41). An OD_{600} of 0.5 was estimated to have 2 x 10^8 bacteria per ml of culture. In
175 experiments examining the peripheral conversion of OVA-specific CD4^{+} T cells to Foxp3^{+}
176 Tregs, subsets of mice were gavaged with 10^{12} attenuated *Salmonella* in PBS one day after
177 congenic cell adoptive transfer. In the helminth-infection experiments, subsets of *H. polygyrus*
178 *bakeri*-infected and uninfected mice were given 2 to 6 x 10^{10} attenuated *Salmonella* in PBS
179 intragastrically using a 20-gauge ball-tipped feeding needle at different time points (14 and 21

180 days after parasite inoculation for antibody production experiments, and 7 days after parasite
181 inoculation for cellular immune response experiments). To determine CFU *Salmonella* per gram
182 tissue, spleens were weighed, homogenized in Hanks Balanced Salt Solution and plated on LB
183 plates containing 100 μg/ml ampicillin.

184 **Intramuscular immunizations.** Mice were injected as previously described (46) with some
185 modifications. 25 μg OVA (Grade V, Sigma) in 1 mg alum or alum alone was suspended in 100
186 μL 1X PBS. 50 μL per limb was injected in the right and left hind leg ventral muscles 14 and 21
187 days after *H. polygyrus bakeri* inoculation.

188 **Helminth infection.** *Heligmosomoides polygyrus bakeri* (*H. polygyrus bakeri)* was propagated
189 as previously described (47) and stored at 4°C until used. C57BL/6J mice were inoculated
190 intragastrically with 200 third-stage larvae using a ball-tipped feeding needle. Adult worms in the
191 intestinal contents were determined at sacrifice as previously described (47).

192 **OVA-TCR transgenic CD4$^+$ T cell enrichment and adoptive transfer in helminth-infected**
193 **and helminth-free mice.** Spleens and MLN were harvested from OT-II (Thy1.1) mice and T
194 lymphocytes were enriched using nylon wool fiber columns (Polysciences, Inc., Warrington,
195 PA). CD4$^+$ T cells were positively selected with CD4 (L3T4) magnetic microbeads (Miltenyi
196 Biotec, Auburn, CA), pooled and suspended in PBS, and 4 to 6 x 10^6 cells injected intravenously
197 into C57BL/6 mice.
198
199 **Flow cytometric analysis.** For the peripheral CD4$^+$ to Foxp3$^+$ Treg experiments,
200 single-cell suspensions from MLN were prepared by passing tissue through a 70-μm cell strainer.
201 For lamina propria (LP) cells, small intestinal segments were incubated in medium containing

3% FCS and 20 mM Hepes (HyClone) for 20 min at 37°C with continuous stirring. Tissue was then digested with 250 mg/ml liberase CI (Roche) and 500 mg/ml DNase I (Sigma-Aldrich), with continuous stirring at 37°C for 30 min. Digested tissue was forced through a Cellector tissue sieve, (Bellco Glass, Inc.) and strained through 70- and 40-μm cell strainers. To enrich for lymphocytes, the suspension was centrifuged at room temperature at 500 *g* for 20 min in 30% Percoll (GE Healthcare) in RPMI-1640. Cells were incubated with antibodies to Ly5.2 (clone 104), CD4 (clone RM4-5), CD25 (clone 7D4), CD103 (clone 2E7; all from eBioscience) and assessed for the expression of these markers in addition to eGFP by flow cytometry using an LSRII (BD Biosciences). Cells were also incubated with mAb against $\alpha_4\beta_7$ (clone DATK32; BD Biosciences), CD44 (IM7; eBioscience), and 7-amino-actinomycin D (7-AAD; BD Biosciences) to detect dead cells. Cells were acquired with an LSR II flow cytometer (BD Biosciences) and flow cytometry data analyzed with FlowJo software (Tree Star, Ashland, OR).

For helminth-infection experiments, Thy1.1 FITC (clone OX-7), CD69 PE (clone H1.2F3), CD4 PerCP (clone RM4-5) and CD25 APC (clone PC61) and isotype controls were purchased from BD Biosciences. Non-specific binding was blocked with antibodies against CD16/CD32 (BD Biosciences, San Jose, CA). For intracellular cytokine staining MLN cells were stimulated as previously described (41) with some modifications. 2 x 10^6 cells/ml were incubated for 24 h with 200 μg/ml ovalbumin protein (OVA, Grade V, Sigma, St.Louis, MO). Prior to being added to cultures, endotoxin levels in the OVA preparation were reduced to less than 0.7 EU/mg using a Detoxi-Gel endotoxin removal column (Pierce, Rockford, IL). During the final 4 h of culture, cells were pulsed with 12.5 ng/ml PMA (Sigma), 500 ng/ml ionomycin (Sigma), and 1 μg/ml GolgiPlug (BD Biosciences). Cells were harvested, surface stained and permeabilized with

Cytofix/Cytoperm Buffer (BD Biosciences), washed with Perm/Wash Buffer (BD Biosciences) and stained with anti-IFN-γ APC (clone XMG1.2) and anti-IL-4 PE (clone 11B11) or anti-IL-10 APC (clone JES5-16E3), and anti-IL-13 PE (clone eBio13A, eBioscience, San Diego, CA). Cells were acquired using a FACScalibur (BD Biosciences) and data analyzed using FlowJo software (Tree Star, Ashland, OR).

Measurement of serum and fecal antibody levels. Sera were collected weekly over the course of each experiment and feces were collected at sacrifice. Sera from individual mice were assayed for OVA-specific IgG1, IgG2b, IgG2c, and IgA by ELISA as previously described (27). For OVA-specific IgG1, IgG2b, and IgG2c, OD values were converted to ng/ml by comparison with a standard curve of anti-OVA Abs affinity purified from the serum of immunized C57BL/6 J mice using OVA conjugated to CNBr-activated Sepharose 4B (Amersham Biosciences, Uppsala, Sweden). To obtain ng/ml values of each anti-OVA Ab isotype, known amounts of purified mouse isotype control Abs from Southern Biotechnology Associates, Birmingham, AL (for IgG1 and IgG2b) or Bethyl Laboratories, Montgomery, TX (for IgG2c) were used. For OVA-specific IgA, OD values were converted to ng/ml of IgA by comparison with a purified IgA standard (BD Biosciences, San Jose, CA). Fecal extracts from individual mice were obtained as previously described (41) and OVA-specific IgA responses were determined by ELISA.

Statistical analysis. Results are expressed as the mean ± standard error of the mean (SEM). One-way ANOVA followed by Tukey's multiple comparisons test, unpaired t tests, or the Mann Whitney test were used to determine the significance of differences among helminth-free and helminth-infected vaccinated and unvaccinated groups of mice. Statistical differences were

247 determined using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). A *P* value of

248 <0.05 was considered significant.

249 **Figure design.** Figures were created using BioRender (https://biorender.com).

RESULTS

A live attenuated oral Salmonella*-OVA vaccine disrupts antigen-specific regulatory networks to promote T-effector responses to OVA in the gut-associated lymphoid tissue*

To determine the impact of oral vaccination with *Salmonella*-OVA on the development of OVA-specific regulatory T cells (Tregs) in the GALT, we introduced *Salmonella*-OVA into a congenic adoptive transfer model previously used to show that oral consumption of dietary OVA antigen drives conversion of OVA-specific T cells into $Foxp3^+$ Tregs in the small intestinal lamina propria (LP) and gut-associated lymphoid tissue (GALT) (44). We adoptively transferred $Ly5.2^+$ T cells from recombination-activating gene 1–deficient (RAG1 KO) OT-II transgenic (OT-II Tg) mice into $Ly5.1^+$ RAG-1-replete B6.SJL recipients. Some recipients were then fed OVA antigen dissolved in drinking water (OVA-water) for five consecutive days. Others were orally vaccinated once with 10^{12} *Salmonella*-OVA or a sham live attenuated *Salmonella* vaccine strain that does not express OVA (*Salmonella*-BEM). Another subset received both OVA-water for five days and one dose of *Salmonella*-OVA (**Fig. 1A**). All $CD4^+$ T cells in $Ly5.2^+$ RAG-1 KO OT-II transgenic mice are specific for OVA and nearly all these $CD4^+$ cells lack expression of the Treg transcription factor Foxp3 (Foxp3-expressing cells <0.05%) (44).We found that oral administration of OVA-water or one dose of *Salmonella*-OVA, but not the sham vaccine, increased the proportions of OVA-specific T cells in the GALT, particularly in the MLN (**Fig. 1B and C**). While OVA-specific T cells accumulated in the intestinal LP of mice fed OVA-water, whether or not they were vaccinated with *Salmonella*-OVA, oral vaccination with *Salmonella*-OVA induced a significantly lower frequency of OVA-specific T cells accumulating in the intestinal LP (**Fig. 1D and E)**.

As expected, soluble OVA (OVA-water) induced the conversion of OVA-specific $CD4^+$ T cells to $Foxp3^+$ Tregs in MLN and intestinal LP ((44) and **Fig. 2**). However, oral vaccination with *Salmonella*-OVA impaired soluble OVA-driven conversion of OVA-specific $CD4^+$ T cells to $Foxp3^+$ Tregs in both the MLN and intestinal LP (**Fig. 2**). Moreover, oral vaccination with *Salmonella*-OVA increased the frequency of activated, OVA-specific, $Foxp3^-$ effector T cells that expressed gut-homing surface molecules α4β7 and CD44 in both MLN (**Fig. 3A-D**) and LP **(Fig 3E-H**). *Salmonella*-OVA attenuated the increase in the frequency of α4β7- and CD44-expressing, OVA-specific, $Foxp3^+$ Tregs in the MLN and LP normally induced by soluble OVA (**Fig. 3**). These data demonstrate that the GALT handles OVA expressed by a live attenuated oral *Salmonella* vaccine in a manner distinct from soluble OVA in drinking water and suggests that OVA acts as a vaccine antigen when introduced in the context of the *Salmonella*-OVA oral vaccine.

Preexisting intestinal helminth infection reduces Th1-skewed OVA-specific antibody responses to oral vaccination with* Salmonella-*OVA

Chronic helminth infection of at least two weeks duration results in significant impairment of host immune responses to Th1/IFN-γ inducing malaria infection (26), IL-12 /IFN-γ dependent trinitrobenzenesulfonic acid (TNBS)-induced colitis (48), and parenteral vaccination against yellow fever virus YFV-17D (22). To determine whether chronic intestinal helminth infection could alter antibody responses to oral immunization, C57BL/6 mice were infected or not with the natural murine gastrointestinal helminth, *H. polygyrus bakeri* 14 days prior to oral vaccination with *Salmonella*-OVA or the sham vaccine *Salmonella*-BEM (**Fig. 4A**). We examined OVA-specific antibody responses to *Salmonella*-OVA or sham vaccine in sera and

fecal extracts of helminth-infected and helminth-free mice (**Fig. 4B-F**). As expected, mice vaccinated with *Salmonella*-BEM did not make OVA-specific antibody responses ((41) and **Fig. 4**). Helminth-free mice vaccinated with *Salmonella*-OVA made a highly Th1-skewed OVA-specific serum IgG2c response, 10 to 15-fold greater than the OVA-specific serum IgG2b and IgG1 responses, respectively (**Fig. 4B-D** and (41)). OVA-specific IgG2b and IgG2c levels were significantly lower in vaccinated helminth-infected mice compared to vaccinated helminth-free mice (**Fig. 4C, D**). Helminth infection delayed but did not eliminate the OVA-specific serum IgA response to *Salmonella*-OVA (**Fig. 4E**). OVA-specific fecal IgA responses were reduced two-fold in helminth-infected vaccinated mice compared to helminth-free mice although this did not reach statistical significance (**Fig. 4F**).

Preexisting helminth infection did not enhance OVA-specific IgG1 responses in orally vaccinated mice (**Fig. 4B**) despite the Th2 polarized helminth-induced polyclonal serum and fecal IgG1 and serum IgE responses in helminth-infected mice (**Supplementary Fig. 1B-D**). We found significantly elevated levels of total serum IgE in helminth-infected mice vaccinated with *Salmonella* compared to their unvaccinated, helminth-infected counterparts, perhaps due to enhanced polyclonal B cell activation in the presence of *Salmonella* LPS, as reported in *in vitro* studies by others (49). The mean number of parasites recovered at sacrifice from the intestinal contents of mice given the sham vaccine *Salmonella*-BEM was lower than that recovered from unvaccinated helminth-infected mice, although this did not reach statistical significance (**Supplementary Fig. 1E**). There was no difference in mean number of parasites recovered from mice vaccinated with *Salmonella*-OVA compared to unvaccinated helminth-infected mice (**Supplementary Fig. 1E**).

Intestinal helminth infection reduces OVA-specific IgG responses to intramuscular vaccination with OVA and the non-microbial adjuvant alum

To determine whether intestinal helminth infection could suppress vaccine antigen-specific antibody responses to a parenterally-administered model OVA protein subunit vaccine, we inoculated C57BL/6 mice with helminth 14 days prior to intramuscular (i.m.) vaccination with OVA adsorbed to the vaccine adjuvant alum (OVA-alum, **Fig. 5A**). We observed that helminth-free mice vaccinated i.m. with OVA-alum made a highly Th2-skewed OVA-specific serum IgG1 response (**Fig. 5B**). Th1-dependent OVA-specific serum IgG2c was not detected and OVA-specific IgG2b levels were 150-fold lower than the OVA-IgG1 levels (data not shown). Notably, helminth-infected mice vaccinated i.m. with OVA-alum made significantly lower OVA-specific IgG1 (**Fig. 5B**) and IgG2b (data not shown) responses when compared to helminth-free vaccinated mice despite elevated, Th2-skewed, polyclonal serum IgG1 and IgE levels (**Fig. 5C, D**). Comparable numbers of adult worms could be recovered from the intestinal contents of both vaccinated and unvaccinated helminth-infected mice (**Fig. 5E**). Th2-skewed antibody responses to i.m. OVA-alum vaccination remained significantly reduced in helminth-infected mice, despite robust polyclonal IgG1 and IgE responses associated with helminth infection, even when mice were housed in a specific pathogen free facility in a completely different institution than in **Fig. 5** (see **Supplementary Fig. 2**). Thus, chronic intestinal helminth infection impaired immune responses to vaccines delivered via either mucosal or parenteral routes.

Helminth infection does not reduce splenic bacterial titers and oral* Salmonella *does not alter helminth-induced organomegaly.

Since SL3261, the parent strain of *Salmonella*-BEM and *Salmonella*-OVA, is highly attenuated, its ability to replicate *in vivo* is limited; however, after intragastric administration, bacteria disseminate systemically and are recoverable from the spleens three days after vaccination and up until four weeks later (data not shown). To determine whether helminth-mediated suppression of antibody responses to oral *Salmonella*-OVA was due to alterations in the systemic dissemination of the vaccine, we examined bacterial titers in the spleens of helminth-infected and uninfected mice three days after oral vaccination (**Supplementary Fig. 3A**). We found no difference in CFU per gram tissue recovered from the spleens of helminth-infected and helminth-free mice vaccinated with *Salmonella*-OVA or *Salmonella*-BEM (**Supplementary Fig. 3B**), suggesting that intestinal helminth infection did not alter systemic trafficking of the live attenuated vaccines. Conversely, ten days after *H. polygyrus bakeri* infection (three days after oral vaccination), comparable numbers of adult worms could be recovered from the intestinal contents of both vaccinated and unvaccinated mice (**Supplementary Fig. 3C**). Both the draining MLN and spleens of helminth-infected mice were enlarged compared to helminth-free mice and significantly greater in mass, regardless of whether the mice were vaccinated with *Salmonella*-BEM or *Salmonella*-OVA (**Supplementary Fig. 3D, E**). Taken together, these data suggest that helminth infection does not impair systemic spread of the *Salmonella* vaccines.

Activated vaccine antigen-specific CD4$^+$ T cells accumulate in the draining MLN of both helminth-free and helminth-infected mice vaccinated with* Salmonella-*OVA

Cytokines produced by antigen-activated CD4$^+$ helper T cells typically drive antibody class switching and stimulate B cells to produce antibodies against T-cell dependent protein

363 antigens (50). To determine if impaired antigen-specific humoral responses in helminth-infected
364 mice were due to a defect in the response to vaccine antigen by antigen-specific $CD4^+$ T helper
365 cells, we adoptively transferred C57BL/6 mice (whose T cells express the surface marker
366 Thy1.2) with $CD4^+Thy1.1^+$ OVA-specific T cell receptor transgenic OT-II cells. Two days later,
367 a subset of mice were infected with helminth larvae. Following helminth infection, mice were
368 orally vaccinated with either *Salmonella*-OVA or the sham vaccine *Salmonella*-BEM (**Fig. 6A**).
369 Three days after vaccination, both the frequency and total number of OVA-specific
370 $CD4^+Thy1.1^+$ OT-II cells in the MLN were higher in helminth-free and helminth-infected mice
371 vaccinated with *Salmonella*-OVA compared to *Salmonella*-BEM vaccinated mice (**Fig. 6B-D**).
372 The mean frequency, but not mean total number, of MLN OT-II cells was significantly lower in
373 helminth-infected, *Salmonella*-OVA vaccinated mice than in their uninfected, *Salmonella*-OVA
374 vaccinated counterparts (**Fig. 6C, D**). This was likely due to a helminth-induced influx of
375 helminth-specific effector cells into the MLN, reflected in the larger organ mass in helminth-
376 infected vaccinated mice (**Supplementary Fig. 3D**) and increased total cell numbers in the
377 draining MLN of helminth-infected mice (data not shown). However, the proportion and total
378 number of OT-II cells that expressed CD69, a marker of early lymphocyte activation, were
379 higher in both helminth-free and helminth-infected mice vaccinated with *Salmonella*-OVA
380 compared to mice vaccinated with *Salmonella*-BEM (**Fig. 6E, F**).

381 Both activated T-effector and Treg populations can express IL-2Rα chain, CD25 (51).
382 We found a modest increase in the percentage, and a significant increase in the number, of
383 $CD25^+$ OT-II cells found in MLNs from helminth-free mice vaccinated with *Salmonella*-OVA
384 compared to helminth-free mice that received the sham vaccine (**Fig. 6G, H**). In addition, the
385 percentage of $CD25^+$ OT-II cells in helminth-infected, vaccinated mice was nearly 2-fold greater

than in helminth-free mice (**Fig. 6G**). However, there was no significant difference in total number of $CD25^+$ OT-II cells recovered from the MLNs of helminth-infected mice vaccinated with *Salmonella*-OVA compared to helminth-free mice (**Fig. 6H**). By contrast, total numbers of MLN, non-TCR transgenic, $CD4^+Thy1.1^-CD25^+$ cells were 2-fold greater in helminth-infected, vaccinated mice than in helminth-free mice (**Supplementary Fig. 4**), suggesting that helminth infection increased total numbers of activated effector and regulatory T cells in orally vaccinated mice.

Helminth-induced Th2-polarized cytokine responses are intact in orally vaccinated mice

Intestinal helminth infection promotes the production of Th2 effector cytokines, including IL-4 and IL-13 (52), and regulatory cytokines like IL-10 and TGF-β (53) by $CD4^+$ T cells. We examined cytokine responses in polyclonal and OVA-specific $CD4^+$ T cell populations following oral vaccination in helminth-free and helminth-infected mice using intracellular staining and flow cytometry (**Fig. 7**). Ten days after helminth infection and three days after oral vaccination, we found comparable frequencies of IFN-$\gamma^+CD4^+Thy1.1^-$ non-TCR transgenic Th1 cells in the draining MLN among helminth-infected and helminth-free mice (**Fig. 7B and C**). However, a significantly greater percentage of $CD4^+Thy1.1^-$ cells were IL-4^+ (**Fig. 7D**), IL-10^+ (**Fig. 7E**), and IL-13^+ (**Fig. 7F**) in both vaccinated and unvaccinated, helminth-infected mice compared to uninfected mice. Thus, oral vaccination with the Th1-polarizing attenuated *Salmonella* vaccine did not prevent the generation of a robust cell-mediated Th2 and Treg cytokine response to helminth infection.

Vaccine antigen-specific CD4$^+$ T cells from the draining MLN of helminth-infected, orally vaccinated mice produce Th2-type effector and regulatory cytokines

We next examined Th1 (IFN-γ), Th2 (IL-4, IL-13), and Treg (IL-10) cytokines in OVA-specific CD4$^+$Thy1.1$^+$ OT-II MLN cells re-stimulated with OVA *in vitro* (**Fig. 8**). The frequencies and total numbers of OT-II cells recovered from cultured MLN cells of helminth-free and helminth-infected *Salmonella*-OVA vaccinated mice were significantly greater than in helminth-free, *Salmonella*-BEM vaccinated mice (**Fig. 8C, D**). Although the percentage and total numbers of IL-13$^+$ and IFN-γ$^+$ OT-II cells were not statistically significantly different between helminth-free and helminth-infected vaccinated mice, the percentage and total numbers of OT-II cells producing, IL-4, and IL-10 was significantly increased in helminth-infected, vaccinated mice compared to uninfected, vaccinated mice (**Fig. 8E-L**). The helminth-modified Th2 response is characterized by elevated antigen-specific Th2-type cytokine production in conjunction with elevated antigen-specific IL-10 production to heterologous antigens administered to helminth-infected mice (54). The increased frequency and total number of Th2-type IL-4$^+$ OT-II cells coupled with enhanced percentages and total numbers of OVA-specific cells producing IL-10 was consistent with the development of a helminth-modified Th2 and Treg response to oral vaccination with *Salmonella*-OVA. The increased frequency and total number of OVA-specific CD4$^+$ IL-10-producing T cells and the drop in serum antibody responses to OVA in helminth-infected, vaccinated mice suggests a role for IL-10-secreting CD4$^+$ T regulatory cells in reducing vaccine-induced humoral responses in helminth-infected mice.

DISCUSSION

In humans and mice, protective immune responses against oral *Salmonella* vaccines involve both T and B cell responses, including robust expansion of $CD4^+$ Th1 cells, IFN-γ production (17, 18), and *Salmonella*-specific IgG and IgA responses that facilitate clearance of the organism (18, 19). We and others have shown that the host immune response to heterologous vaccine antigen produced within the recombinant attenuated oral *Salmonella* vaccine (RASV) system is a $CD4^+$ Th1-biased immune response (41, 55) that depends on intact signaling via MyD88 (41). The experiments presented here expand our current understanding of how the RASV system induces immunity to heterologous vaccine antigens. We show that the RASV system disrupts vaccine antigen-specific regulatory T cell networks in the gut-associated lymphoid tissue (GALT). It reduces the frequency of activated, vaccine-antigen specific, $Foxp3^+$ regulatory T cells in the GALT that express gut-homing markers. The RASV system concurrently promotes the accumulation of activated, vaccine antigen-specific, $Foxp3^-$ effector T cells expressing gut-homing surface molecules in the GALT (**Figs. 2 and 3**).

Because they are versatile and reliably induce Th1-biased immune responses, live attenuated oral *Salmonella* vaccine strains are widely used in agriculture, veterinary medicine, and preventative care of humans to protect against salmonellosis (11, 12), typhoid (13, 14) and paratyphoid fever (15). The recombinant attenuated oral *Salmonella* vaccine (RASV) system has also been used as an experimental vaccine platform to develop oral vaccine candidates for protection against food borne parasites (56, 57), human papilloma virus (58), streptococcal pneumonia (55), and shigellosis (59), among many other pathogens. Yet, disparities in the immunogenicity of oral *Salmonella* vaccines (60, 61) and other oral and parenteral vaccines in

low- and middle-income countries compared to high-income countries have been repeatedly described (62-65).

One compelling hypothesis for this disparity is that endemic helminth infection alters immune responses to vaccination, and indeed, in human population studies, multiple reports describe decreased vaccine efficacy in people with chronic helminth infections (60, 66-69). In this study, we used a murine model to examine the impact of intestinal helminth infection on the response to vaccination. We demonstrated that chronic infection with the intestinal helminth *Heligmosomoides polygyrus bakeri* significantly suppressed Th1-skewed OVA-specific antibody responses to our live attenuated oral *Salmonella*-OVA vaccine (**Fig. 4)**. Strikingly, despite robust helminth-induced Th2-biased total IgG1 and IgE responses in helminth-infected, vaccinated mice (**Supplementary Fig. 1**), *H. polygyrus bakeri* infection failed to enhance the development of a Th2-dependent OVA-specific IgG1 response to *Salmonella*-OVA (**Fig. 4**).

The reduced antibody responses to oral vaccination in helminth-infected mice were not due to an impaired ability of the live attenuated *Salmonella* to traffic systemically and reach immune organs like the spleen. By day 3 after oral vaccination, comparable CFU *Salmonella* per gram tissue were recoverable from the spleens of helminth-free and helminth-infected mice (**Supplementary Fig. 3**). The reduced antigen-specific humoral responses were also not due to impaired ability of adaptive immune cells in helminth-infected mice to recognize and respond to vaccine antigens. OVA-specific $CD4^+$ T cells expressing the activation marker CD69 accumulated in the draining MLN of both helminth-free and helminth-infected mice vaccinated with the OVA-expressing *Salmonella* (**Fig. 6**). Moreover, similar numbers of OVA-specific $CD4^+$ T cells in helminth-infected and helminth-free mice vaccinated with *Salmonella*-OVA produced the Th1 effector cytokine IFN-γ when re-stimulated *in vitro* with OVA (**Fig. 8**).

Vaccination with *Salmonella*-OVA did not alter helminth-induced organomegaly (**Supplementary Fig. 3**) nor did it hinder the development of Th2-polarized cytokine responses in $CD4^+$ T cells from vaccinated mice (**Fig. 7**). Notably, helminth infection primed for a Th2-biased and Treg-biased cytokine response to an ordinarily Th1-biasing vaccine, inducing greater frequencies of IL-4 and IL-10-producing vaccine antigen-specific $CD4^+$ T cells (**Fig. 8**). The elevation in Th2 and Treg cytokines that we observed, in both polyclonal and vaccine antigen-specific T cell populations, mirrors the helminth-modified Th2 response to heterologous antigens previously reported by Mangan and colleagues in a mouse model of allergen-induced airway disease with concomitant helminth infection (54). This signature cytokine pattern has been observed in a variety of allergic and inflammatory disease models in our lab and others (29, 48). Helminth-induced IL-10 production in particular has been implicated in protecting against both chemically-induced, colonic inflammation (48, 70) and allergic inflammation (29, 71). While the helminth-modified, Th2 cytokine response is beneficial and protective in these inflammatory disease models, our data suggest that it is detrimental for generating robust immune responses in our helminth infection/vaccine model. IL-10-secreting, $CD4^+$ T cell populations are associated with helminth-mediated immune suppression, as are $CD4^+CD25^+$ T cells, even in the absence of IL-10 secretion (72). Accordingly, we found an increased frequency of polyclonal (**Fig. 7**) and vaccine-antigen specific (**Fig. 8**) $CD4^+$ IL-10-producing T cells and higher numbers of polyclonal $CD4^+CD25^+$ T cells (**Supplementary Fig. 4**) in helminth-infected compared to helminth-free mice.

Helminth-induced alterations in vaccine antigen-induced cytokine production have been previously described in both human studies and mouse models (31, 67, 69, 73). Elias et al. observed reduced purified protein derivative (PPD)-specific IFN-γ secretion by peripheral blood

497 mononuclear cells isolated from bacilli Calmette-Guerin (BCG)-vaccinated Ethiopian subjects
498 with concomitant intestinal helminth infection when compared to anthelmintic-treated controls
499 (69). Su et al. and Nookala et al. both reported decreased vaccine antigen-induced IFN-γ in their
500 models, with enhanced production of *P. chabaudi* antigen-specific IL-4, IL-13, and IL-10 in an
501 intestinal helminth infection/malaria vaccination model (31) and enhanced tetanus toxoid-
502 specific IL-10 in the human lymphatic filariasis/tetanus vaccination study (73). Surprisingly, we
503 found no difference in the frequencies of polyclonal IFN-γ^+CD4$^+$ T cells between helminth-free
504 and helminth-infected mice (**Fig. 7**). There were also comparable frequencies and total numbers
505 of vaccine antigen-specific IFN-γ^+ CD4$^+$ T cells in helminth-infected and helminth-free
506 vaccinated mice (**Fig. 8**). This may reflect the potent Th1-inducing properties of our live
507 attenuated *Salmonella* vaccine compared to the protein subunit plus adjuvant vaccines employed
508 in the other studies.

509 Intestinal helminth infection induced OVA-specific, Th2 cytokine-producing CD4$^+$ T
510 cells after oral *Salmonella*-OVA vaccination, but this did not translate into enhanced vaccine
511 antigen-specific, Th2-dependent IgG1 antibody production in helminth-infected mice. Even in
512 the context of intramuscular vaccination with OVA adsorbed to the adjuvant alum, which
513 promotes Th2-skewed antibody responses to co-administered protein antigens (74, 75), intestinal
514 helminth infection suppressed Th2-dependent, antigen-specific IgG1 production (**Fig. 5** and
515 **Supplementary Fig. 2**). Intestinal helminth infection has been shown to modulate cellular
516 immune responses to i.m. and intravenous vaccination in mice (31, 32). Chronic co-infection
517 with multiple viral pathogens in conjunction with the intestinal helminth *H. polygyrus bakeri* can
518 reduce serum antibody responses to subcutaneous injection of the yellow fever vaccine (22).

519 Our study highlights the suppressive effect of intestinal helminth infection on vaccine antigen-
520 specific antibody responses to i.m. vaccination, even in the absence of any other infection.
521 The discordance of the impact of helminth-infection on vaccine-induced, T-effector cell
522 responses compared to humoral immune responses (76) may depend on worm burden and
523 chronicity of helminth infection, as has been shown in epidemiologic studies examining the
524 effects of helminth infection on allergic disease (33). Individuals with chronic helminth infection
525 and heavy worm burdens in a Venezuelan study were protected from atopic skin reactivity
526 against house dust mite antigen, whereas those with sporadic infection and light worm burdens
527 had elevated allergen-specific IgE responses and high skin reactivity (77). Su et al. have reported
528 a similar phenomenon in their malaria/intestinal helminth coinfection model; while *H. polygyrus*
529 *bakeri* infection of one week duration could suppress antimalarial immunity and increase levels
530 of parasitemia, infection of two weeks or longer exacerbated malaria-induced morbidity and
531 resulted in mortality in C57BL/6 mice (26). In our vaccination model, we found that chronic
532 nematode infection of at least two weeks duration suppressed anti-OVA antibody responses to
533 *Salmonella*-OVA (**Fig. 4**). Although *H. polygyrus bakeri* infection is confined to the small
534 intestines, infection with this helminth alters gut microbial communities across the small and
535 large intestines, and within the feces (39, 78, 79). These alterations in gut microbial
536 communities, including enrichment in members of the order Clostridiales (39, 79) and elevated
537 levels of their associated metabolic products, i.e. short chain fatty acids, have a significant
538 impact on heterologous systemic immune responses (39) and likely contribute to helminth-
539 mediated suppression of vaccine-antigen responses in our model.
540 We demonstrate that intestinal infection with *H. polygyrus bakeri* generated MLN-
541 resident, polyclonal and vaccine antigen-specific, $CD4^+$ T cells that produced the regulatory

cytokine IL-10 (**Figs. 7** and **8**). Infection with *H. polygyrus bakeri* has been shown to promote the expansion of $Foxp3^{+}CD4^{+}CD25^{+}$IL-10 producing regulatory T cells by producing a TGF-β mimic that can induce regulatory T cells *in vitro* even in the presence of inflammatory cytokines (80, 81). Helminth glycans have also been shown to drive regulatory T cell expansion in mixed type 2 / regulatory T cell responses characterized by the presence of IL-10-producing regulatory T cells (82). Chronic parasitic infection in mice with the systemic, blood-borne, filarial helminth *Litmosomoides sigmodontis* induces an expansion of splenic, $Foxp3^{-}$ IL-10^{+}, T regulatory 1 (Tr1) cells and reduces the quantity and quality of influenza vaccine-specific antibody responses (83). In an environmental enteric dysfunction model comprised of severely malnourished mice chronically infected with adherent *E. coli*, LP-resident $Foxp3^{+}ROR\gamma T^{+}$Tregs were associated with impaired antibody responses to an oral heat labile toxin vaccine (84). Our data demonstrate that neither a systemic chronic infection, nor severe malnutrition is required for the expansion of infection-associated regulatory T cells and suppressed vaccine-specific antibody responses. We show that in well-nourished hosts, a strictly enteric chronic helminth infection promoted the expansion of IL-10-producing T cells and impaired antibody responses to both injectable and live attenuated oral vaccines.

Our findings confirm that helminth-induced alteration of the intestinal microenvironment has systemic consequences, in this case, down-modulating immune responses to parenteral and oral vaccination. Antigen-specific antibody responses to different vaccine formulations (live attenuated *Salmonella* vaccine and protein adsorbed to alum) administered via different routes (oral and intramuscular) were suppressed by preexisting intestinal helminth infection. Our findings suggest that the immune suppressive environment generated by intestinal helminths to promote their survival impacts “third party” immune responses that may hinder the development

565 of vaccine-induced protective immunity. Thus, the potential need to eliminate these parasites
566 prior to vaccination should be considered when targeting populations with endemic helminth
567 infection. In fact, large-scale clinical trials in a helminthic-endemic area (Uganda) have recently
568 been proposed to investigate whether anti-helminthic therapies will enhance antibody and T-
569 effector cytokine responses to both injectable and oral vaccines in school age children (61).
570 Future studies exploring $CD4^+$T-cell independent mechanisms and their possible contributions to
571 helminth-induced suppression of vaccine-induced antibody responses is also warranted. Our data
572 make clear that the optimization of vaccine schedules in helminth-endemic regions must take
573 into account that even strictly enteric helminth infection alters local mucosal and systemic
574 immune responses to vaccination.
575

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CONFLICT OF INTEREST

Cathryn R. Nagler is the President and Co-Founder of ClostraBio, Inc. Onyinye Iweala is a consultant for Blueprint Medicines and Novartis. The other authors declare no conflicts of interest.

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dysfunction induces regulatory T cells that inhibit local CD4+ T cell responses and impair oral vaccine efficacy. *Immunity* 54: 1745-1757 e1747.

867 **FIGURE LEGENDS**

868 **Figure 1. Feeding with soluble OVA antigen and/or oral vaccination with *Salmonella*-OVA**
869 **induces accumulation of OVA-specific T cells in the GALT.** (A) Experimental timeline. After
870 gating on $CD4^+$ T cells, transferred T cells in the MLN (B, C) or LP (D, E) of OVA antigen-fed
871 mice or mice vaccinated with *Salmonella*-OVA (*Salm*-OVA) or the sham vaccine *Salmonella*-
872 BEM (*Salm*-BEM) were identified by Ly5.2 expression. B and D are representative flow
873 cytometry plots while C and E are summary graphs of the percentage of $Ly5.2^+$ RAG1 KO OT-II
874 T cells in MLN and LP respectively. Each dot in C and E represents a single mouse, with three
875 to four mice per group. Experiment was repeated two times. Data shown are from one of two
876 independent experiments. Statistical Analyses: One-way ANOVA followed by Tukey's multiple
877 comparisons test (****P<0.0001, ***P<0.001, **P<0.01).
878
879 **Figure 2. Oral vaccination with *Salmonella*-OVA disrupts oral soluble OVA-driven**
880 **conversion of $CD4^+$ T cells to $Foxp3^+$ Tregs in the GALT.** After gating on $CD4^+$ T cells,
881 transferred T cells in the MLN (A, B) or LP (C, D) of OVA antigen-fed or *Salmonella*-OVA
882 (*Salm*-OVA) vaccinated mice were identified by Ly5.2 expression. $Ly5.2^+$ cells were then
883 assessed for intracellular Foxp3 expression. A and C are representative flow cytometry plots
884 while B and D are summary graphs of the percentage of $Foxp3^+$ among $Ly5.2^+$ RAG1 KO OT-II
885 T cells in MLN and LP respectively. Each dot in B and D represents a single mouse with four
886 mice per group. Experiment was repeated two times. Data shown are from one of two
887 independent experiments. Statistical Analyses: One-way ANOVA followed by Tukey's multiple
888 comparisons test (***P<0.001, **P<0.01, *P<0.05).
889

Figure 3. Oral vaccination with *Salmonella*-OVA increases the frequency of activated, OVA-specific Foxp3$^-$ T effectors and decreases the frequency of OVA-specific Foxp3$^+$ Tregs expressing gut homing molecules in the MLN and lamina propria. After gating on CD4$^+$ T cells, transferred T cells in the MLN of OVA antigen-fed or *Salmonella*-OVA vaccinated mice were identified by Ly5.2 expression. Ly5.2$^+$ cells in the MLN (A-D) and LP (E-H) were then assessed for intracellular Foxp3 expression and cell surface expression of α4β7 (A, B, E, F) or CD44 (C, D, G, H). A, C, E, and G are representative flow cytometry plots while B, D, F and H are summary graphs. Each dot represents a single mouse with four mice per group. Experiment was repeated two times. Data shown are from one of two independent experiments. Statistical Analyses: one-way ANOVA followed by Tukey's multiple comparisons test (***P<0.001, **P<0.01, *P<0.05).

Figure 4. Th1-skewed antibody responses to oral vaccination with *Salmonella*-OVA are reduced in mice with preexisting helminth infection. (A) Experimental timeline; 14 days after intragastric inoculation with 200 third-stage *H. polygyrus bakeri* larvae, helminth-free (B-E; No Inf, black bars) and helminth-infected (B-E; Hp inf, white bars) C57BL/6 mice were given two intragastric doses of 2 x 10^{10} *Salmonella*-BEM (SALM-BEM) or *Salmonella*-OVA (SALM-OVA) and OVA-specific serum (B) IgG1, (C) IgG2b, (D) IgG2c, and (E) IgA were measured 14, 21, and 28 days post vaccination by ELISA. OVA-specific IgA in fecal extracts was measured 28 days post vaccination by ELISA (F). Pooled data from two independent experiments (n=11-12 mice per group). Statistical Analyses: Unpaired t-test in B-E comparing helminth-infected to helminth-free at same time point; One-way ANOVA in F followed by Tukey's multiple comparisons test. ***P<0.001, **P<0.01, *P<0.05.

Figure 5. Th2-skewed antibody responses to intramuscular vaccination with OVA-alum are significantly reduced while polyclonal IgG1 and IgE responses are elevated in vaccinated, helminth-infected mice. (A) Experimental timeline; 14 days after intragastric inoculation with 200 third-stage *H. polygyrus bakeri* larvae, helminth-free C57BL/6 mice (black bars) and helminth-infected C57BL/6 mice (white bars), were given two intramuscular doses of 25 mg OVA adsorbed to 1 mg alum or 1mg alum alone spaced one week apart. (B) OVA-specific IgG1. (C) Total Serum IgG1. (D) Total serum IgE. (E) Worms recovered. Data in B-E are pooled from two independent experiments; n=8-10 mice per group. Statistical Analyses: unpaired t-test in B; One-way ANOVA followed by Tukey's multiple comparisons test in C-E (****P<0.0001, **P<0.01).

Figure 6. Activated vaccine antigen-specific CD4$^+$ T cells accumulate in the draining MLN of both helminth-free and helminth-infected mice vaccinated with *Salmonella*-OVA. (A) Experimental timeline. (B) Representative flow cytometry plots showing adoptively transferred CD4$^+$Thy1.1$^+$ OVA-TCR transgenic OT-II cells, percent CD69$^+$ and percent CD25$^+$ among OT-II cells in MLNs of helminth-free and helminth-infected mice. (C) Proportion of adoptively transferred CD4$^+$Thy1.1$^+$ OVA-TCR transgenic OT-II cells in MLNs of helminth-free (black symbols) and helminth-infected (white symbols) mice. (D) Total number OT-II cells recovered from MLN. (E) Percent CD69$^+$ among OT-II cells. (F) Total number CD69$^+$OT-II cells (G) Percent CD25$^+$ among OT-II cells (H) Total number CD25$^+$OT-II cells. Salm-BEM (circles) = *Salmonella*-BEM. Salm-OVA (squares) = *Salmonella*-OVA. Hp = *H. polygyrus bakeri*. Symbols represent individual mice; lines represent mean percentages. Pooled data from three independent

experiments; n=5 to 7 mice per group. Statistical Analyses: one-way ANOVA followed by Tukey's multiple comparisons test (****P<0.0001, ***P<0.001, **P<0.01, *P<0.05).

Figure 7. Intestinal helminth infection induces Th2-polarized cytokine responses in CD4$^+$ T cell populations in both vaccinated and unvaccinated mice. (A) Experimental timeline; two days after adoptive transfer of 4 to 6 x 10^6 CD4$^+$Thy1.1$^+$ OVA-TCR transgenic OT-II cells, mice were infected (white symbols) or not (black symbols) with 200 third-stage *H. polygyrus bakeri* (Hp) larvae. Seven days after helminth infection, mice received one intragastric dose of ~5 x 10^{10} *Salmonella*-BEM or *Salmonella*-OVA. 3 days later, MLN cells were harvested, cultured overnight with OVA, pulsed for 4 h with PMA, ionomycin, and Golgiplug and surface labeled with mAbs to CD4 and Thy1.1, fixed, permeabilized and intracellularly stained with Abs against IFN-γ, IL-4, IL-10, and IL-13. (B) Representative flow cytometry plots and summary graphs showing (C) percent IFN-γ$^+$ (D) percent IL-4$^+$ (E) percent IL-10$^+$ (F) percent IL-13$^+$ among non-TCR transgenic CD4$^+$Thy1.1$^-$ MLN cells. Salm-BEM (circles) = *Salmonella*-BEM. Salm-OVA (squares) = *Salmonella*-OVA. Hp (triangles) = *H. polygyrus bakeri* only. Symbols represent individual mice; lines represent mean percentages. Pooled data from 3 independent experiments; n=3 to 7 mice per group. Statistical Analyses: One-way ANOVA followed by Tukey's multiple comparisons test (****P<0.0001, ***P<0.001, **P<0.01, *P<0.05).

Figure 8. OVA-specific CD4$^+$ T cells from the draining MLN of helminth-infected mice vaccinated with *Salmonella*-OVA produce Th2 effector and regulatory cytokines (A) Experimental timeline. (B) Representative flow cytometry plot and (C-L) summary graphs showing (C) percent CD4$^+$Thy1.1$^+$ and (D) total number of CD4$^+$Thy1.1$^+$ among re-stimulated

MLN cells (E) percent IFN-γ^+ and (F) total number of IFN-γ^+ (G) Percent IL-4^+ (H) total number IL-4^+ (I) percent IL-10^+ and (J) total number IL-10^+ (K) percent IL-13^+ and (L) total number IL-13^+ of adoptively transferred $CD4^+Thy1.1^+$ OT-II MLN cells. MLN cells were harvested, cultured overnight with OVA, pulsed for 4 h with PMA, ionomycin, and Golgiplug and surface labeled with mAbs to CD4 and Thy1.1, fixed, permeabilized and intracellularly stained with Abs against IFN-γ, IL-4, IL-10, and IL-13. Helminth-free (black symbols); helminth-infected (white symbols). Salm-BEM (circles) = *Salmonella*-BEM. Salm-OVA (squares) = *Salmonella*-OVA. Symbols represent individual mice; lines represent mean percentages. Pooled data from three independent experiments; n=5 to 7 mice per group. Statistical Analyses: one-way ANOVA followed by Tukey's multiple comparisons test (C, D) and Mann Whitney test (E-L. ***P<0.001, **P<0.01, *P<0.05).

Chronic Helminth Infection Impairs Vaccine Immunity

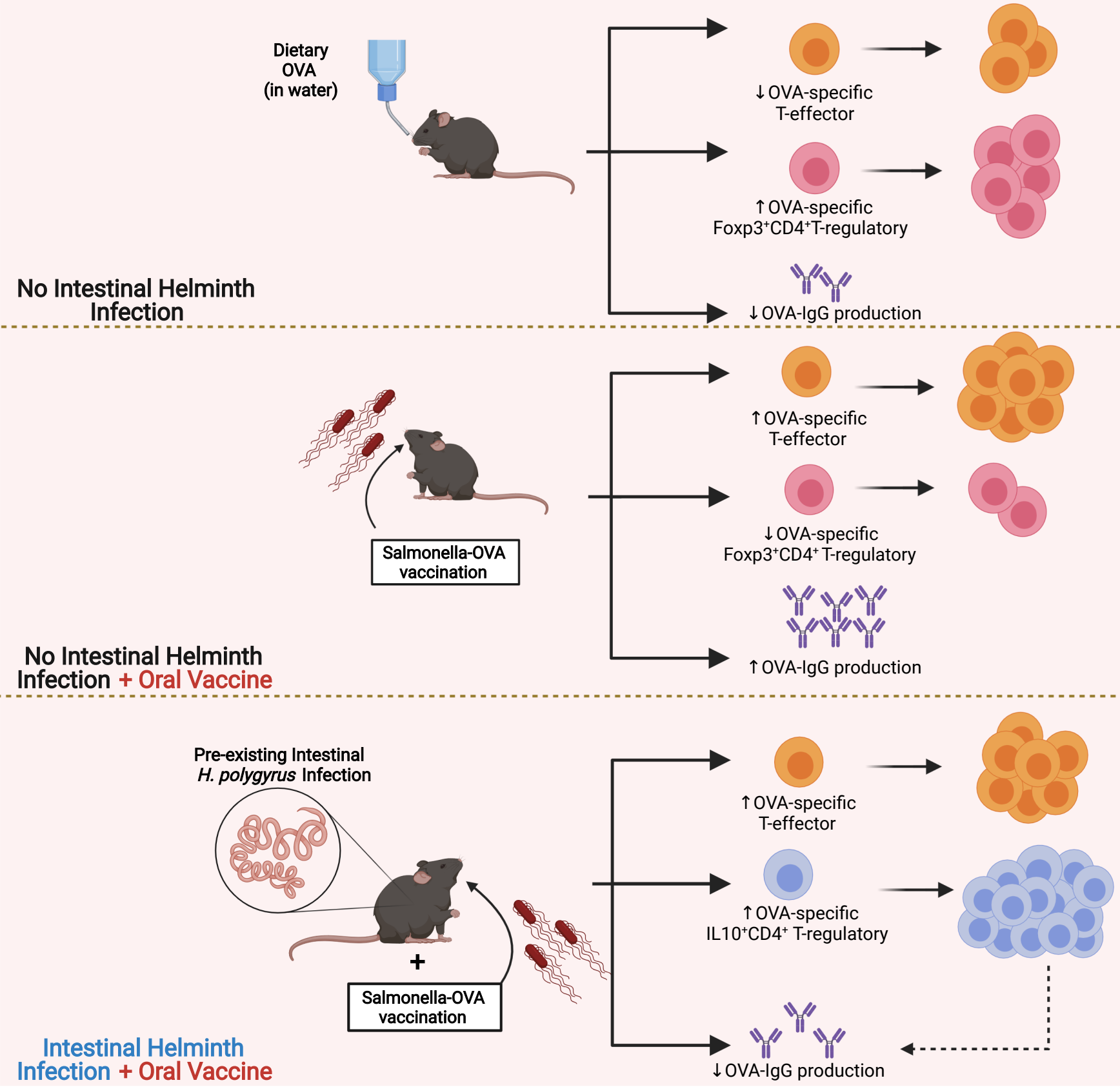


Figure 1

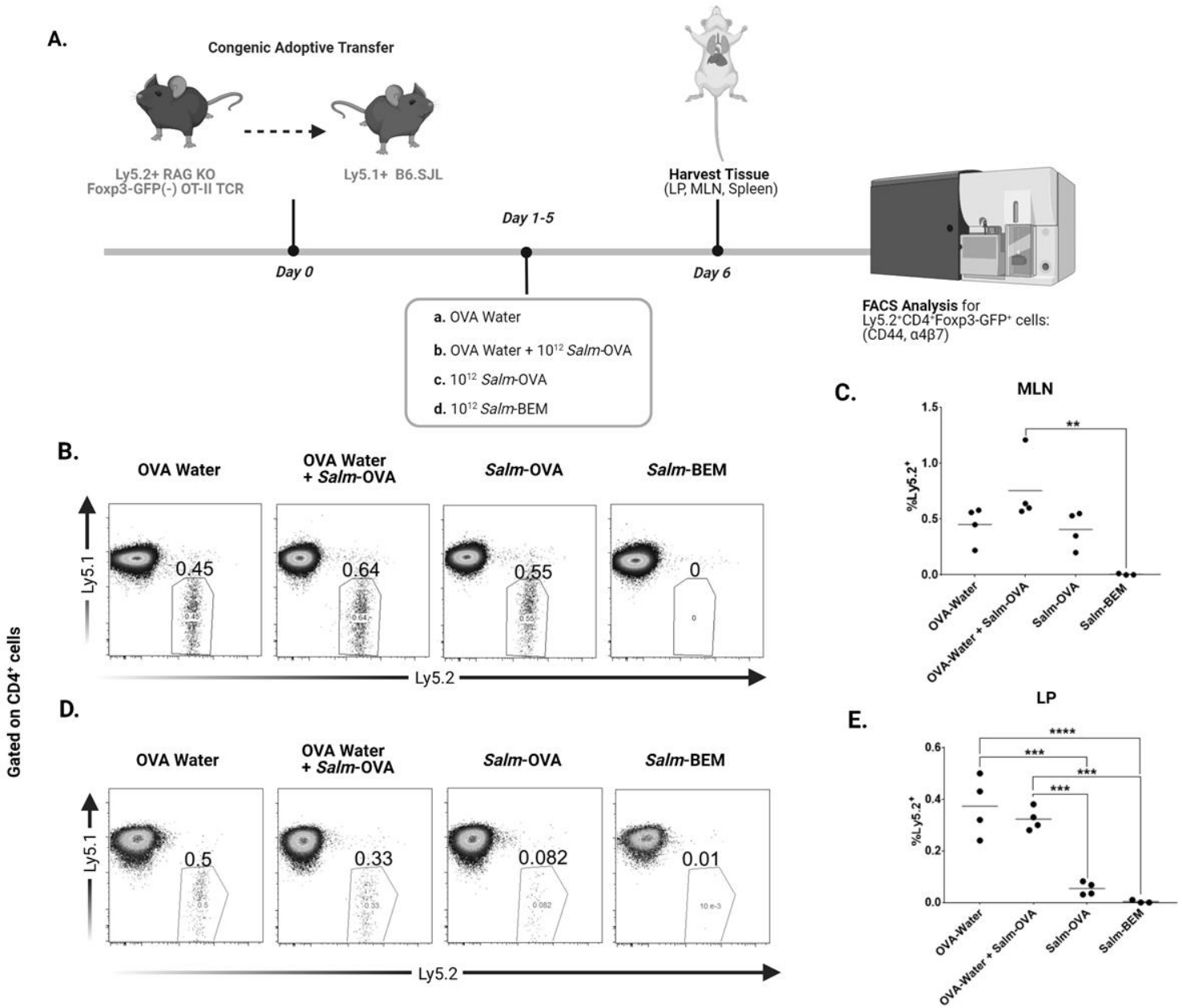

A.
Congenic Adoptive Transfer
Ly5.2+ RAG KO
Foxp3-GFP(-) OT-II TCR
Ly5.1+ B6.SJL
Harvest Tissue
(LP, MLN, Spleen)
Day 0
Day 1-5
Day 6
a. OVA Water
b. OVA Water + 10^12 Salm-OVA
c. 10^12 Salm-OVA
d. 10^12 Salm-BEM
FACS Analysis for
Ly5.2+CD4+Foxp3-GFP+ cells:
(CD44, α4β7)
B.
OVA Water
OVA Water + Salm-OVA
Salm-OVA
Salm-BEM
Ly5.1
Ly5.2
0.45
0.64
0.55
0
Gated on CD4+ cells
D.
0.5
0.33
0.082
0.01
C.
MLN
%Ly5.2+
OVA-Water
OVA-Water + Salm-OVA
Salm-OVA
Salm-BEM
E.
LP

Figure 2

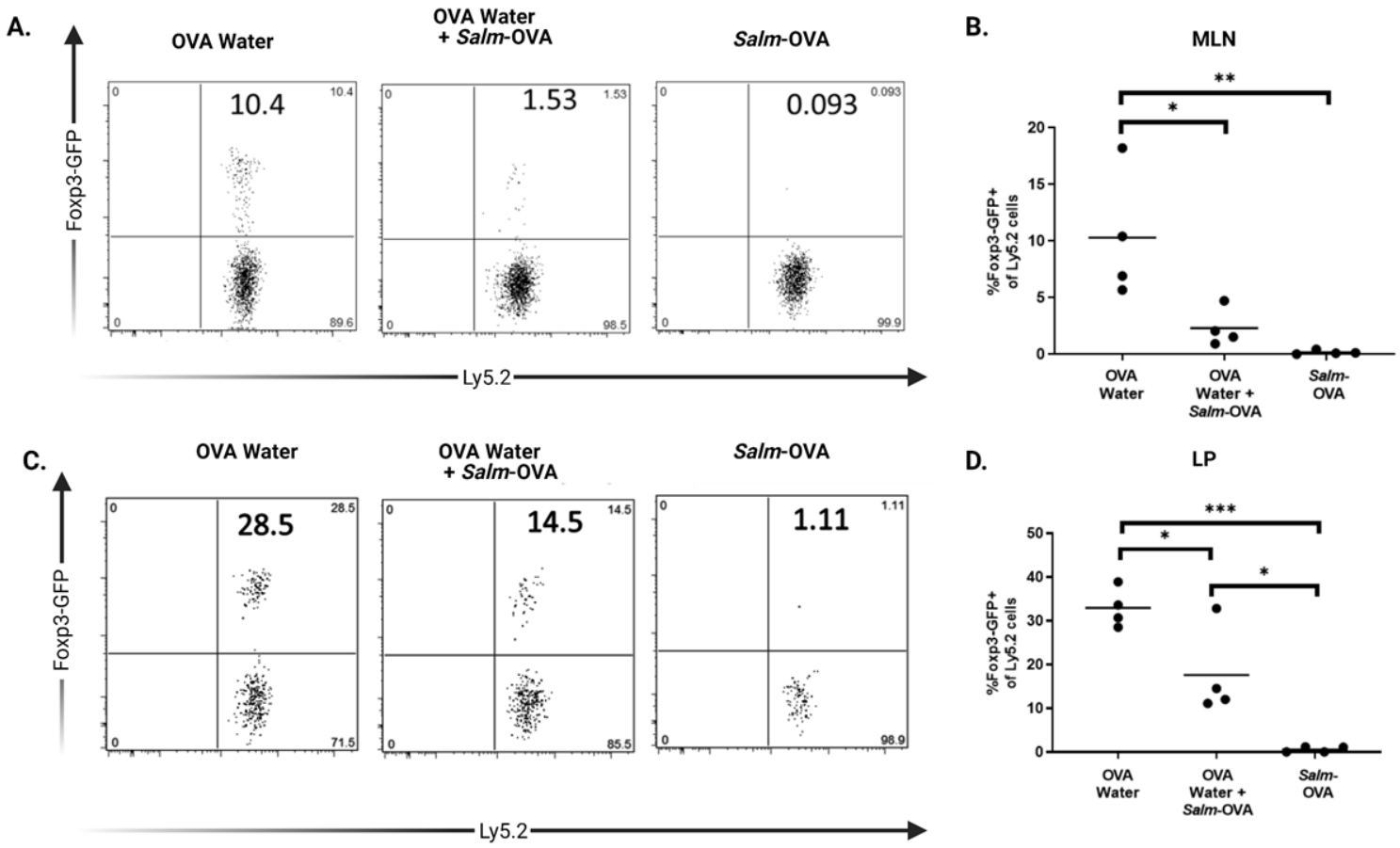

A.
OVA Water
OVA Water + Salm-OVA
Salm-OVA
Foxp3-GFP
Ly5.2
10.4
1.53
0.093
89.6
98.5
99.9
B.
MLN
%Foxp3-GFP+ of Ly5.2 cells
OVA Water
OVA Water + Salm-OVA
Salm-OVA
C.
28.5
14.5
1.11
71.5
85.5
98.9
D.
LP

Figure 3

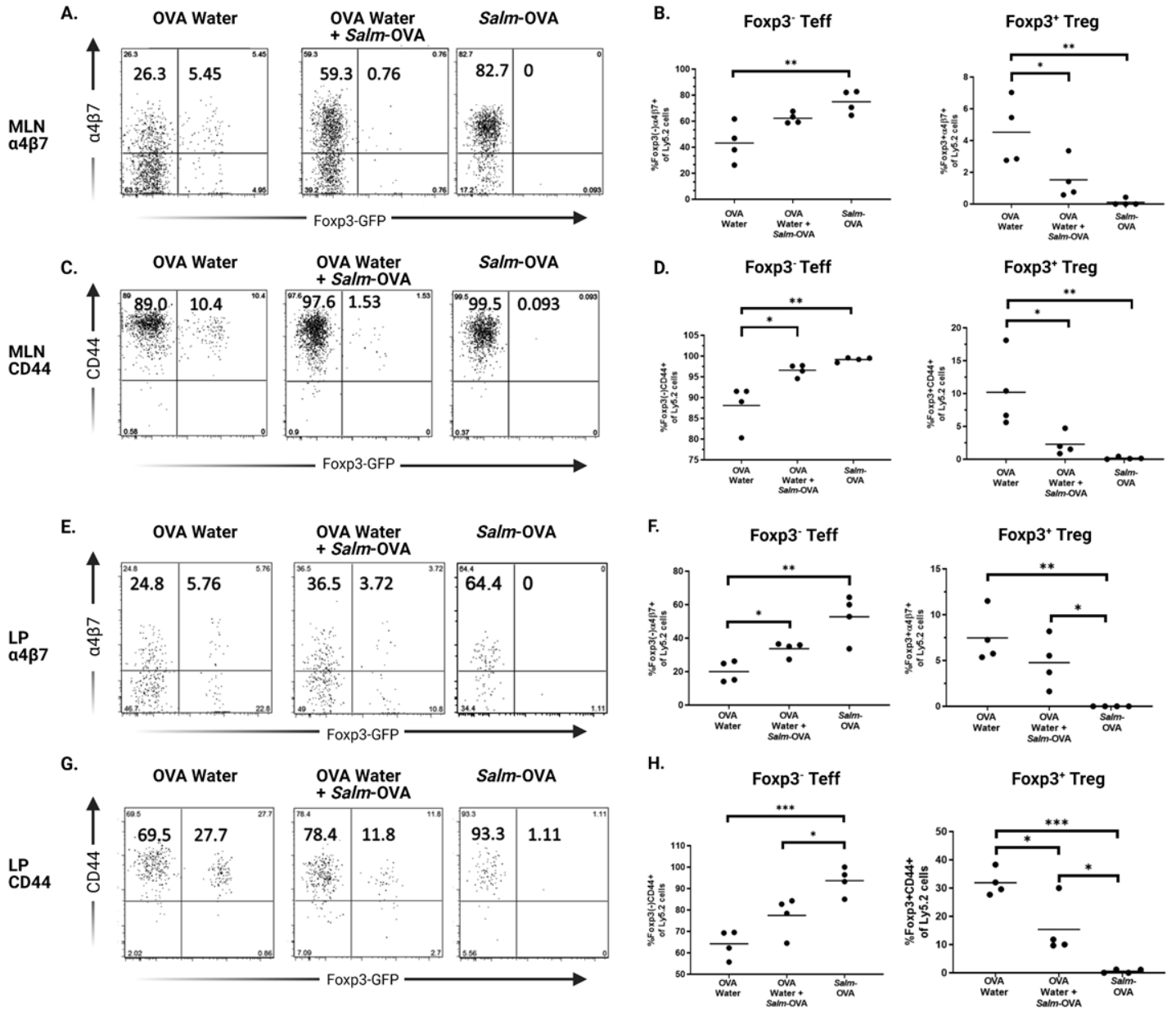

A.
OVA Water
OVA Water + Salm-OVA
Salm-OVA
MLN α4β7
α4β7
26.3
5.45
59.3
0.76
82.7
0
Foxp3-GFP
B.
Foxp3⁻ Teff
Foxp3⁺ Treg
%Foxp3(-)α4β7+ of Ly5.2 cells
%Foxp3+α4β7+ of Ly5.2 cells
OVA Water
OVA Water + Salm-OVA
Salm-OVA
C.
MLN CD44
CD44
89.0
10.4
97.6
1.53
99.5
0.093
D.
%Foxp3(-)CD44+ of Ly5.2 cells
%Foxp3+CD44+ of Ly5.2 cells
E.
LP α4β7
24.8
5.76
36.5
3.72
64.4
0
F.
G.
LP CD44
69.5
27.7
78.4
11.8
93.3
1.11
H.

Figure 4

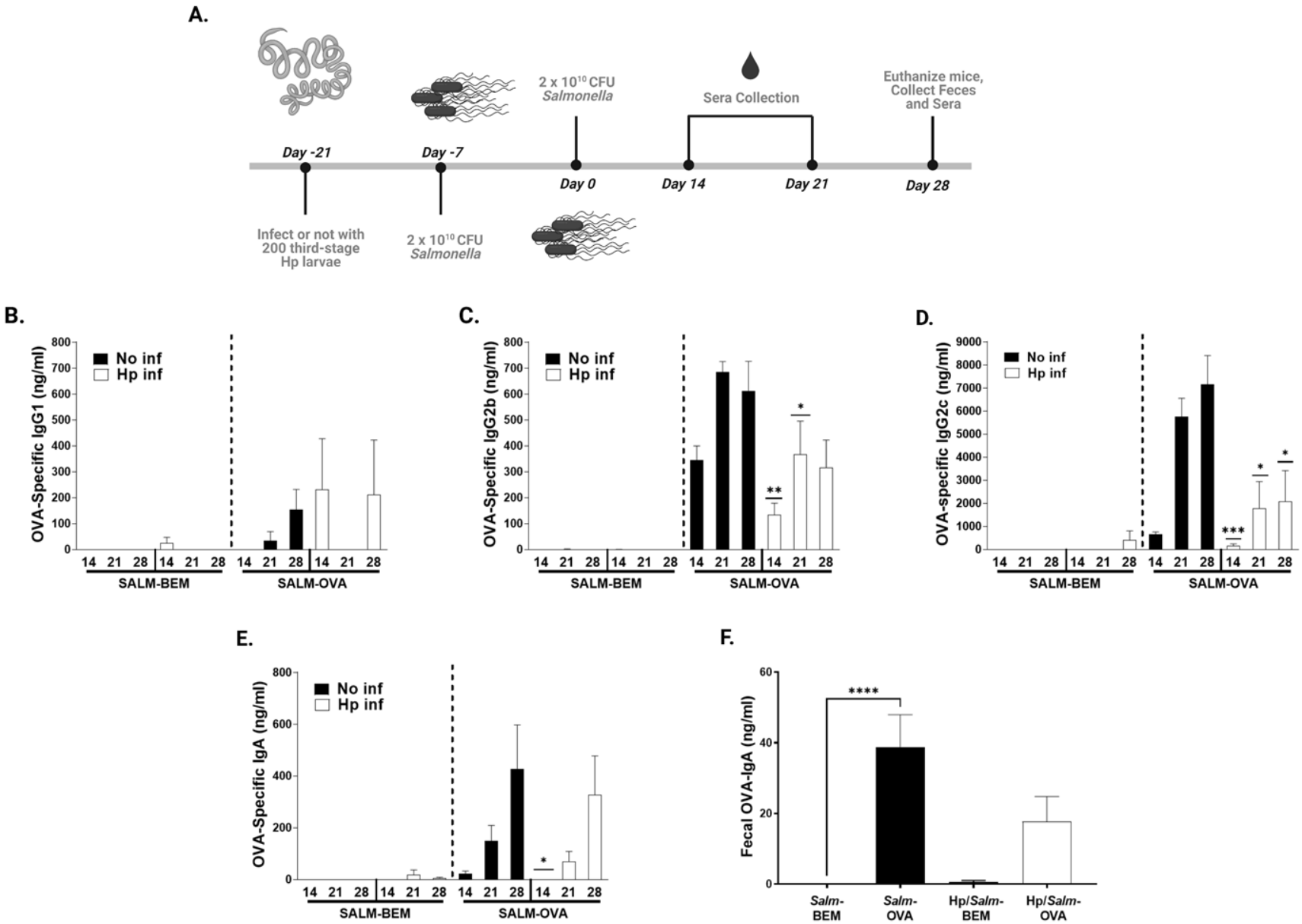

A.
2 x 10^10 CFU Salmonella
Sera Collection
Euthanize mice, Collect Feces and Sera
Day -21
Day -7
Day 0
Day 14
Day 21
Day 28
Infect or not with 200 third-stage Hp larvae
2 x 10^10 CFU Salmonella
B.
OVA-Specific IgG1 (ng/ml)
C.
OVA-Specific IgG2b (ng/ml)
D.
OVA-specific IgG2c (ng/ml)
No inf
Hp inf
14 21 28 14 21 28 14 21 28 14 21 28
SALM-BEM
SALM-OVA
E.
OVA-Specific IgA (ng/ml)
F.
Fecal OVA-IgA (ng/ml)
****
Salm-BEM
Salm-OVA
Hp/Salm-BEM
Hp/Salm-OVA

Figure 5

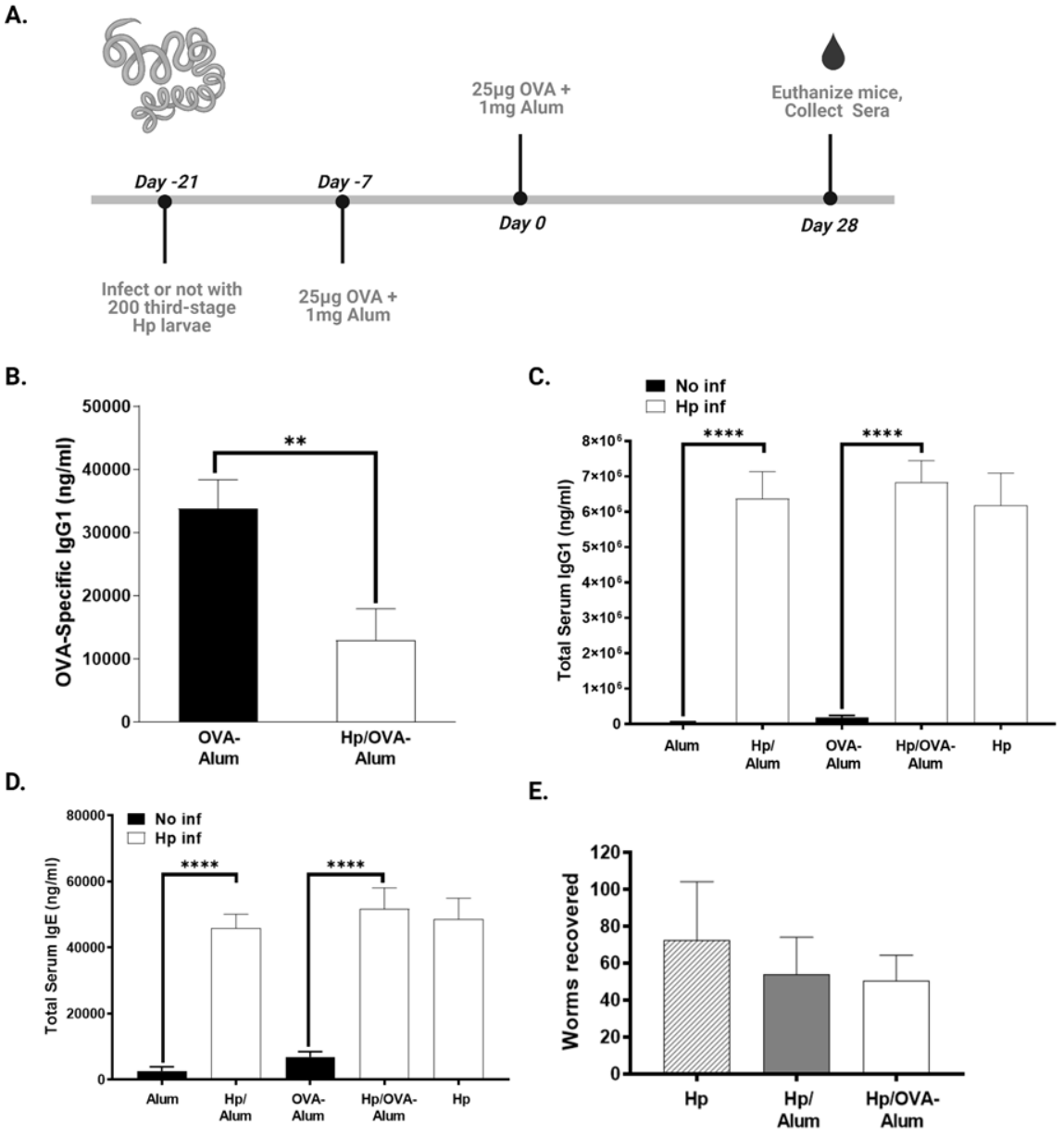

A.
Day -21
Day -7
Day 0
Day 28
Infect or not with 200 third-stage Hp larvae
25µg OVA + 1mg Alum
25µg OVA + 1mg Alum
Euthanize mice, Collect Sera
B.
OVA-Specific IgG1 (ng/ml)
50000
40000
30000
20000
10000
0
**
OVA-Alum
Hp/OVA-Alum
C.
No inf
Hp inf
Total Serum IgG1 (ng/ml)
8×10⁶
7×10⁶
6×10⁶
5×10⁶
4×10⁶
3×10⁶
2×10⁶
1×10⁶
0
****
****
Alum
Hp/Alum
OVA-Alum
Hp/OVA-Alum
Hp
D.
Total Serum IgE (ng/ml)
80000
60000
40000
20000
0
No inf
Hp inf
****
****
Alum
Hp/Alum
OVA-Alum
Hp/OVA-Alum
Hp
E.
Worms recovered
120
100
80
60
40
20
0
Hp
Hp/Alum
Hp/OVA-Alum

Figure 6

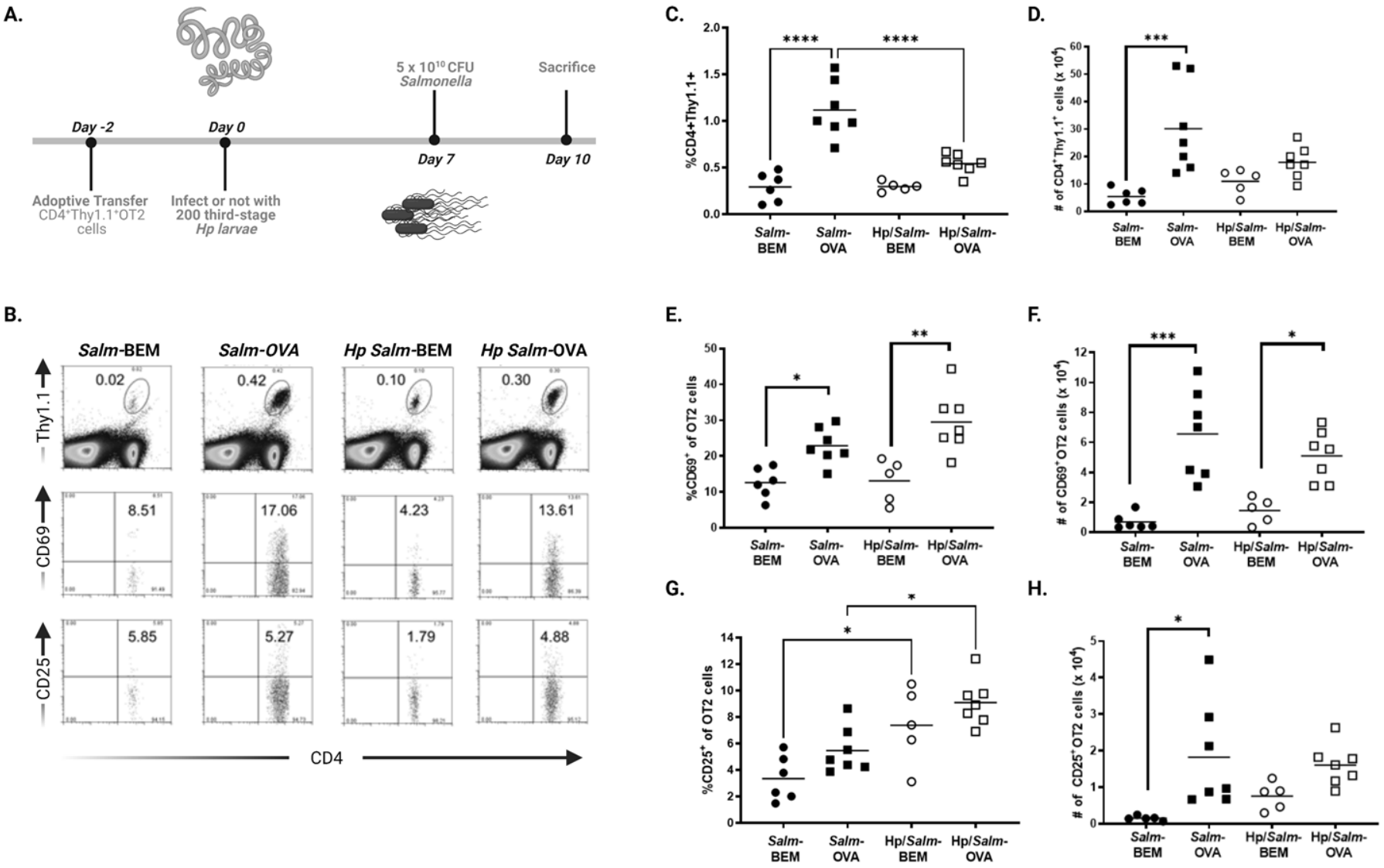

A.
5 x 10^10 CFU
Salmonella
Sacrifice
Day -2
Day 0
Day 7
Day 10
Adoptive Transfer
CD4+Thy1.1+OT2
cells
Infect or not with
200 third-stage
Hp larvae
B.
Salm-BEM
Salm-OVA
Hp Salm-BEM
Hp Salm-OVA
Thy1.1
CD69
CD25
0.02
0.42
0.10
0.30
8.51
17.06
4.23
13.61
5.85
5.27
1.79
4.88
CD4
C.
%CD4+Thy1.1+
****
****
D.
# of CD4+Thy1.1+ cells (x 10^4)
***
E.
%CD69+ of OT2 cells
*
**
F.
# of CD69+OT2 cells (x 10^4)
***
*
G.
%CD25+ of OT2 cells
*
*
H.
# of CD25+OT2 cells (x 10^4)
*
Salm-BEM
Salm-OVA
Hp/Salm-BEM
Hp/Salm-OVA

Figure 7

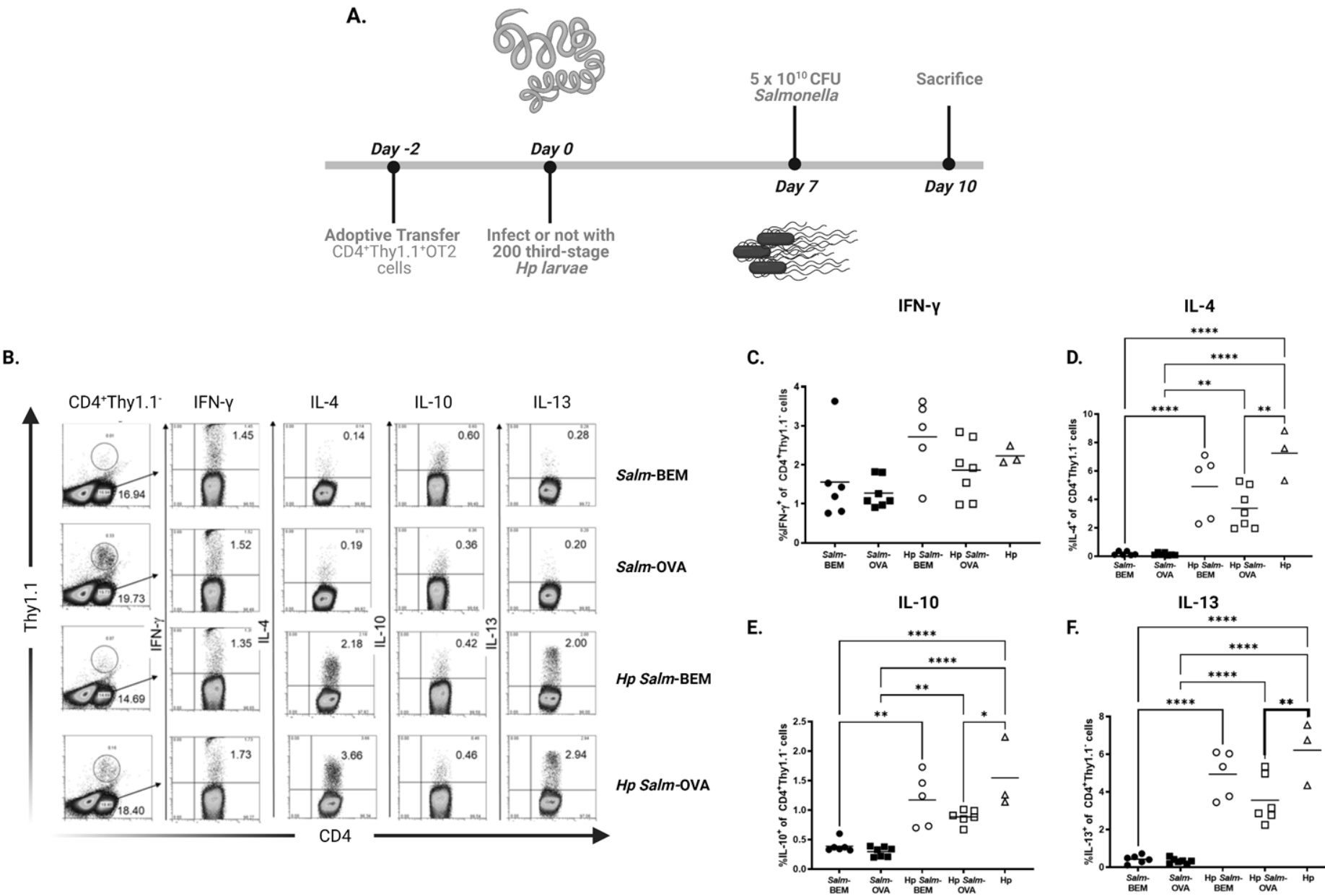
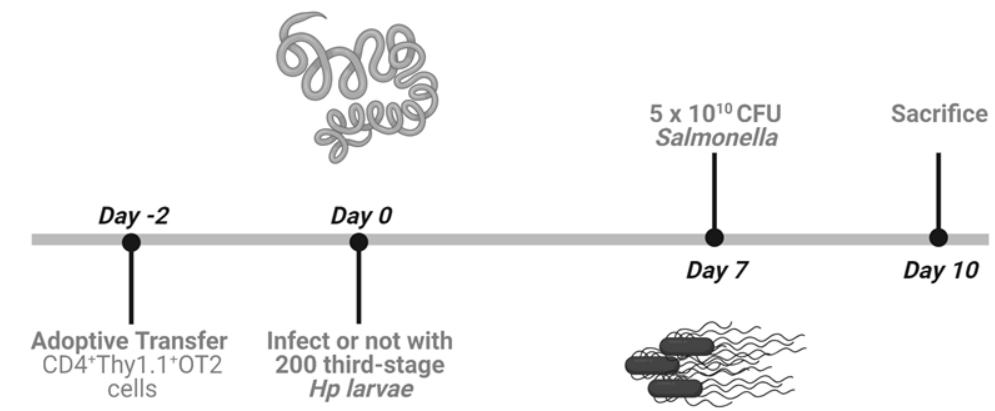
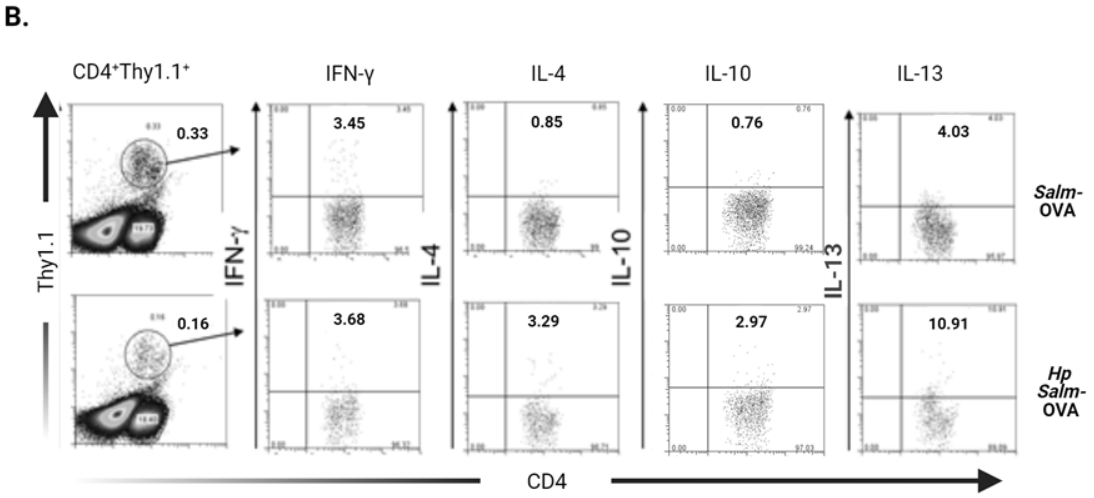
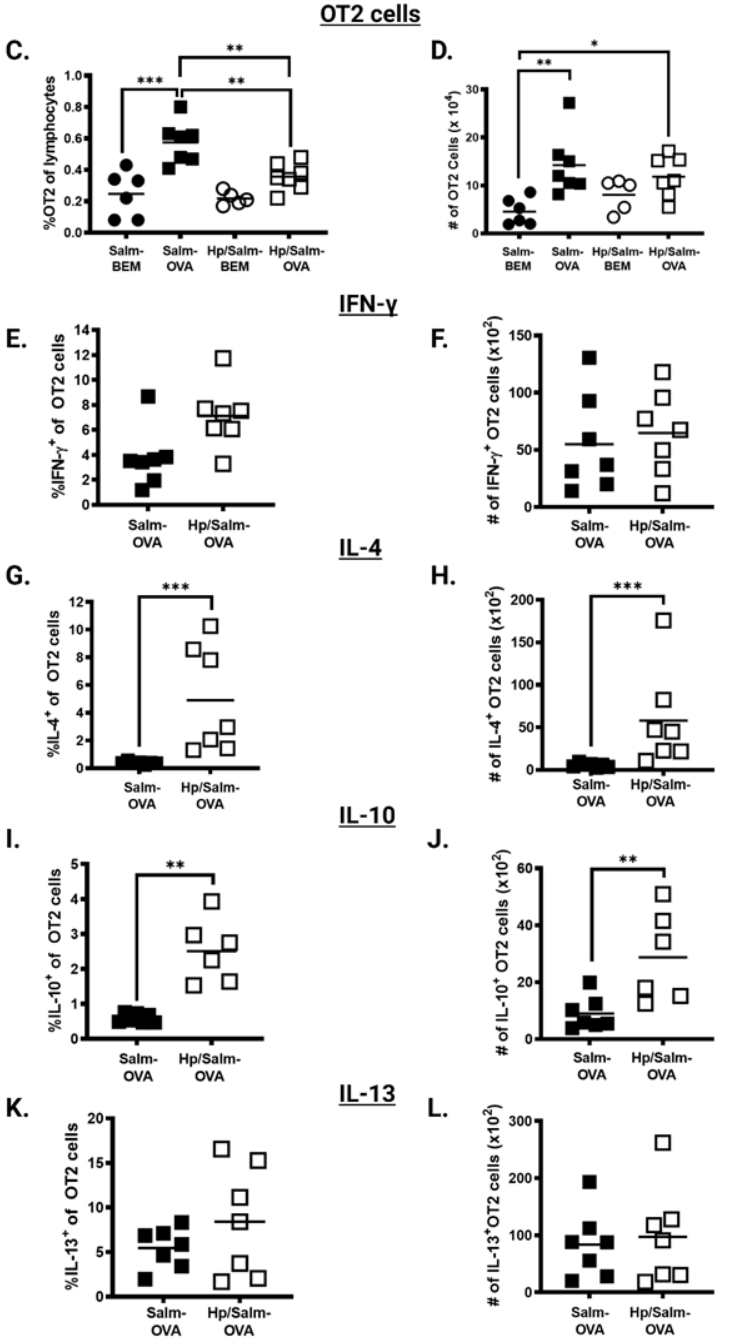

A.
5 x 10[10] CFU
Salmonella
Sacrifice
Day -2
Day 0
Day 7
Day 10
Adoptive Transfer
CD4+Thy1.1+OT2
cells
Infect or not with
200 third-stage
Hp larvae
B.
CD4+Thy1.1-
IFN-γ
IL-4
IL-10
IL-13
Thy1.1
CD4
Salm-BEM
Salm-OVA
Hp Salm-BEM
Hp Salm-OVA
16.94
1.45
0.14
0.60
0.28
19.73
1.52
0.19
0.36
0.20
14.69
1.35
2.18
0.42
2.00
18.40
1.73
3.66
0.46
2.94
C.
IFN-γ
%IFN-γ+ of CD4+Thy1.1- cells
D.
IL-4
%IL-4+ of CD4+Thy1.1- cells
E.
IL-10
%IL-10+ of CD4+Thy1.1- cells
F.
IL-13
%IL-13+ of CD4+Thy1.1- cells
Salm-BEM
Salm-OVA
Hp Salm-BEM
Hp Salm-OVA
Hp

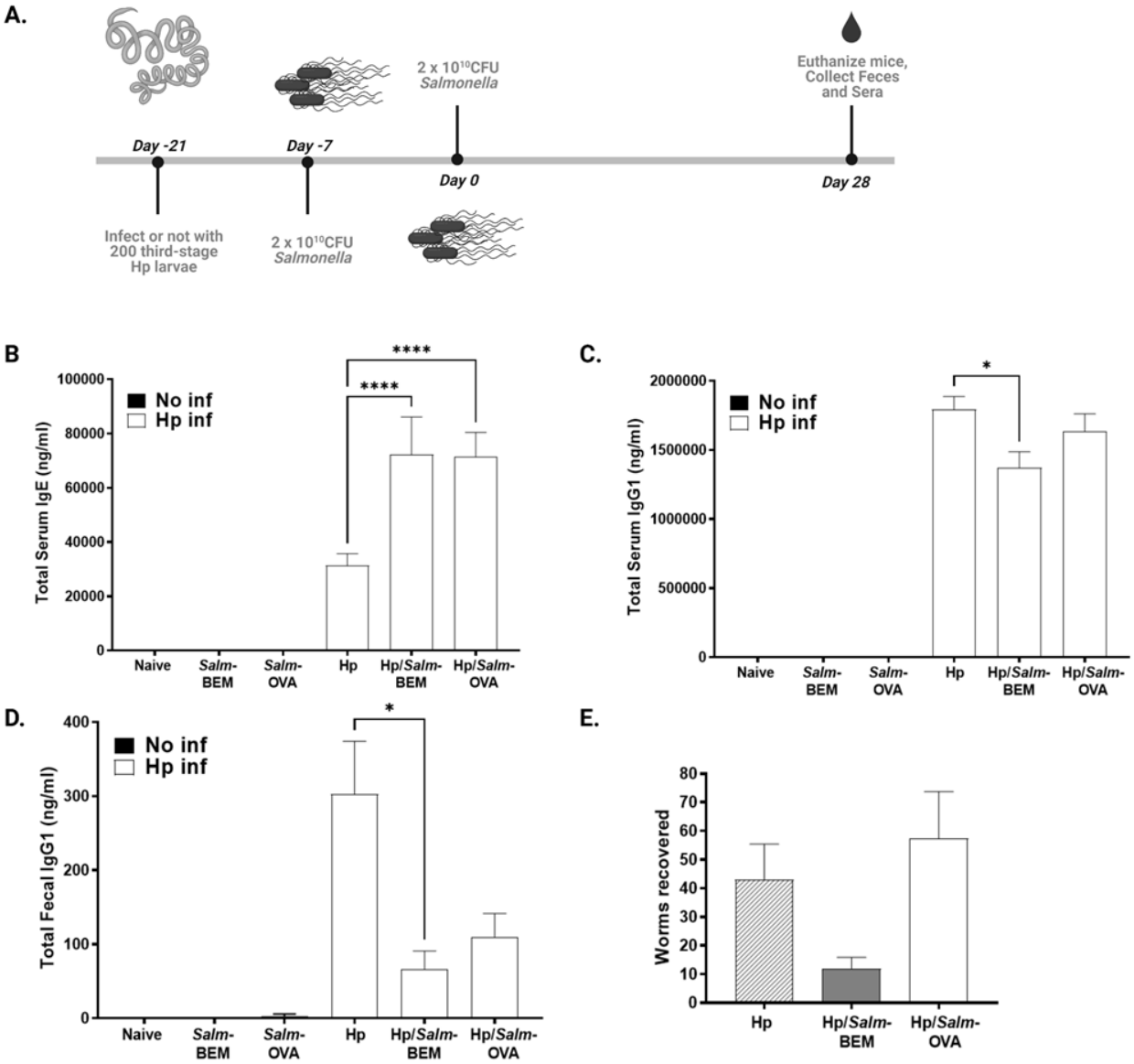
Figure 8


A.
5 x 10¹⁰ CFU
Salmonella
Sacrifice
Day -2
Day 0
Day 7
Day 10
Adoptive Transfer
CD4⁺Thy1.1⁺OT2
cells
Infect or not with
200 third-stage
Hp larvae

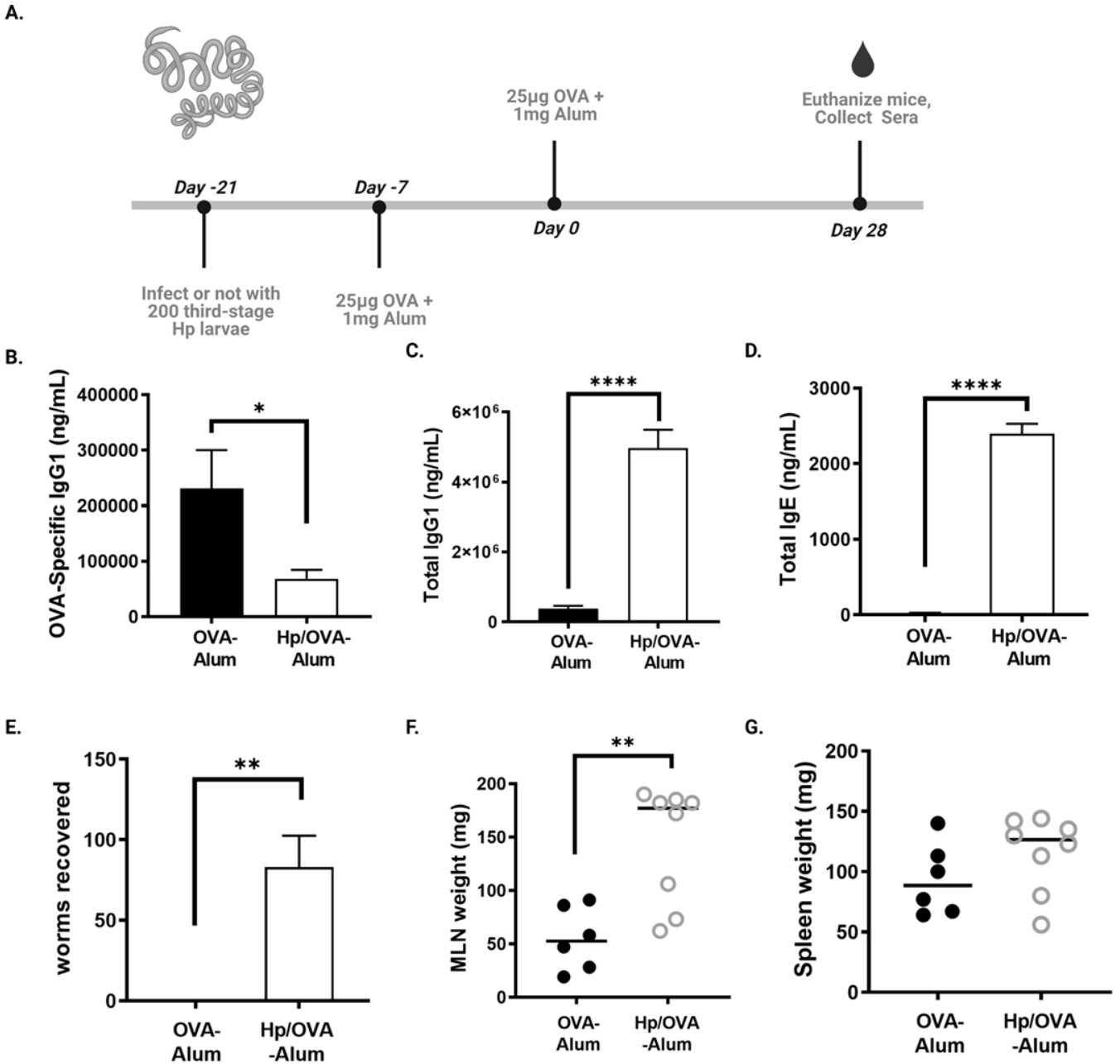

B.
CD4⁺Thy1.1⁺
IFN-γ
IL-4
IL-10
IL-13
0.33
3.45
0.85
0.76
4.03
0.16
3.68
3.29
2.97
10.91
Thy1.1
IFN-γ
IL-4
IL-10
IL-13
CD4
Salm-
OVA
Hp
Salm-
OVA


OT2 cells
C.
%OT2 of lymphocytes
D.
# of OT2 Cells (x 10⁴)
Salm-
BEM
Salm-
OVA
Hp/Salm-
BEM
Hp/Salm-
OVA
IFN-γ
E.
%IFN-γ⁺ of OT2 cells
F.
# of IFN-γ⁺ OT2 cells (x10²)
IL-4
G.
%IL-4⁺ of OT2 cells
H.
# of IL-4⁺ OT2 cells (x10²)
IL-10
I.
%IL-10⁺ of OT2 cells
J.
# of IL-10⁺ OT2 cells (x10²)
IL-13
K.
%IL-13⁺ of OT2 cells
L.
# of IL-13⁺OT2 cells (x10²)

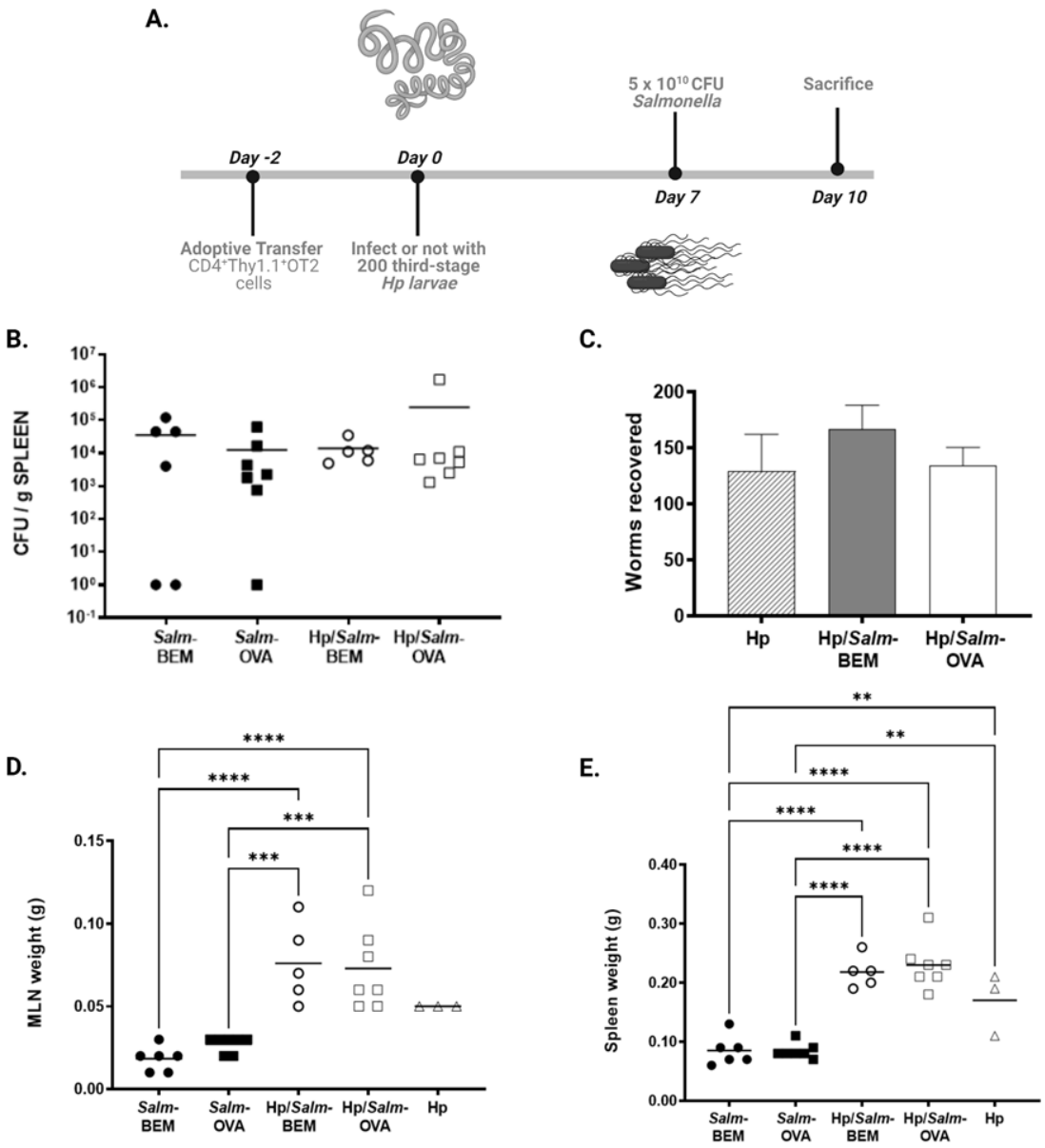
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4