

# IgG and IgE Collaboratively Accelerate Expulsion of *Strongyloides venezuelensis* in a Primary Infection

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**The host deploys a subset of immune responses to expel helminths, which differs depending on the nature of the helminth. *Strongyloides venezuelensis*, a counterpart of the human pathogen *S. stercoralis*, naturally infects rodents and has been used as an experimental model. Here we show that induction of immunoglobulin G (IgG) and IgE is a prerequisite for rapid expulsion of *S. venezuelensis* during a primary infection. Activation-induced cytidine deaminase-deficient (AID<sup>-/-</sup>) mice, which lack the ability to switch IgM to other isotypes, normally developed T-helper 2 (Th2) cells and intestinal mastocytosis after infection with *S. venezuelensis*. Although AID<sup>-/-</sup> mice expelled *Nippostrongylus brasiliensis* normally, they required a much longer period to expel *S. venezuelensis* than wild-type (WT) mice. Adoptive transfers of immune sera from *S. venezuelensis*-infected but not *N. brasiliensis*-infected mice restored the ability of AID<sup>-/-</sup> mice to promptly expel *S. venezuelensis*. Immune serum-derived IgG and IgE induced worm expulsion via Fc $\gamma$  receptor III (Fc $\gamma$ RIII) and Fc $\epsilon$  receptor I (Fc $\epsilon$ RI), respectively, and a mixture of IgG and IgE showed collaborative effects. Whereas Fc $\gamma$ RIII<sup>-/-</sup> mice or Fc $\epsilon$ RI $\alpha$ <sup>-/-</sup> mice normally could expel *S. venezuelensis*, Fc $\gamma$ RIII<sup>-/-</sup> mice, when their IgE was neutralized by anti-IgE, or Fc $\epsilon$ RI $\alpha$ <sup>-/-</sup> mice, when their IgG binding to Fc $\gamma$ RIII was blocked by anti-Fc $\gamma$ RIII, showed a markedly reduced ability to expel *S. venezuelensis*. These data reveal that IgG and IgE play redundant roles but act in concert to accelerate *S. venezuelensis* expulsion. Mast cell-deficient mice, even those equipped with immune serum-derived IgG or IgE, failed to expel *S. venezuelensis* promptly, suggesting that mast cells are cellular targets of IgG and IgE.**

Intestinal helminth infections induce Th2-type immune responses characterized by goblet cell hyperplasia, mastocytosis, eosinophilia, and high serum levels of IgG1 and IgE. The host deploys a subset of these immune responses to expel intestinal helminths, which differs depending on the nature of the helminth. *Nippostrongylus brasiliensis* is a gut-dwelling nematode whose expulsion depends on interleukin-4 (IL-4)/IL-13 produced by Th2 cells. Increased IL-4/IL-13 levels in the intestinal milieu after infection drive goblet cell hyperplasia and smooth muscle cell contraction, leading to interferences of *N. brasiliensis* adherence and survival (1, 2). The presence of B cells and antibody (Ab) production are dispensable for *N. brasiliensis* expulsion during primary and challenge infections (3). In contrast, Ab production is an essential component of the immune responses against particular helminths. The IgE fraction in immune sera is regarded as a major player, because IgE-deficient mice show susceptibility to infections with *Schistosoma mansoni* (4), *Trichinella spiralis* (5, 6), or *Brugia malayi* (7). Administration of the IgG1 fraction from hyperimmune sera causes significant worm reduction in a primary infection with *Heligmosomoides polygyllus bakari* (8). A recent study demonstrated that naturally existing IgG from naive mice suppresses the fecundity of adult worms during a primary *Heligmosomoides polygyllus bakari* infection (9). These results have established the importance of Ig class switching in immune responses against particular helminth infections.

Murine strongyloidiasis has been used as an experimental model for human strongyloidiasis. Human strongyloidiasis is caused by *Strongyloides stercoralis*, which is distributed widely in tropical and subtropical areas. Although most infected people are asymptomatic, immunocompromised individuals could develop

fatal hyperinfection and dissemination. Note that subjects treated with corticosteroids or other immunosuppressants, those coinfecting with human T cell leukemia virus 1, diabetic patients, those with hematologic malignancies, organ transplant recipients, and malnourished infants are vulnerable (10).

The relevance of cellular and humoral immunity has been demonstrated in the host defense mechanism against murine strongyloidiasis induced by *Strongyloides ratti* and *Strongyloides venezuelensis*. A transfer of immune sera or mesenteric lymph node cells from infected mice confers resistance against *S. ratti* to naive wild-type (WT) mice (11). Immune sera exert a protective effect against the early-migrating tissue stage of larvae, and the IgG1-rich fraction shows the greatest protective activity among Ig isotypes (12). In contrast, a transfer of immune sera is insufficient to induce *S. venezuelensis* expulsion in hypothyroid nude mice (13). These findings indicate that immune serum-mediated immunity against *Strongyloides* species is T cell dependent.

Mucosal mast cells in the intestinal wall have been shown to promote expulsion of *S. venezuelensis* (14–16). Proliferation of mucosal mast cells is induced by Th2 cells or their products (IL-3

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and IL-9), and as we demonstrated previously, IL-18 also induces proliferation of mucosal mast cells by driving CD4<sup>+</sup> T cells to produce IL-3 and IL-9 without inducing their development into Th2 cells (17). Thus, IL-3 and IL-9, which are derived from activated Th2 cells and IL-18-stimulated CD4<sup>+</sup> T cells, play crucial roles in intestinal mastocytosis. Taken together, these observations indicate that the T cell dependence of *Strongyloides* species expulsion may be due largely to the T cell-derived growth factors for mucosal mast cells.

Fcε receptor γ chain-deficient (FcRγ<sup>-/-</sup>) mice display intestinal mastocytosis after infection with *S. venezuelensis*. Nevertheless, they exhibit delayed kinetics of worm expulsion (18). This result clearly indicates that mast cell accumulation does not fully explain the immunity against *S. venezuelensis* and that FcRγ-mediated signaling also plays a relevant role in expelling *S. venezuelensis*. FcRγ is a widely expressed adaptor bearing an ITAM that transduces activation signals from various immunoreceptors, including Fc receptors, Ig-like receptors, and C-type lectins (19–21). This broad association precludes a simple interpretation of FcRγ-mediated *S. venezuelensis* expulsion.

We hypothesized that Abs are principal activators of FcRγ expressed on mucosal mast cells in promoting *S. venezuelensis* expulsion. To demonstrate this hypothesis, we employed AID<sup>-/-</sup> mice, which are devoid of Ig class switching. AID<sup>-/-</sup> mice produce more IgM than do WT mice, but they lack IgA, IgG, and IgE (22). Therefore, AID<sup>-/-</sup> mice are suitable recipients for passive transfers of class-switched Abs during the course of *S. venezuelensis* infection.

## MATERIALS AND METHODS

**Mice.** Specific-pathogen-free C57BL/6 mice, BALB/c mice, WBB6F1<sup>+/+</sup> mice, WBB6F1-W/W<sup>v</sup> mice, Wistar rats, and Sprague-Dawley rats were purchased from SLC Japan (Hamamatsu, Japan). C57BL/6 AID<sup>-/-</sup> mice were obtained from Riken BRC through the National Bio-Resource Project of the MEXT, Japan (22). C57BL/6 Fcγ receptor III-deficient (FcγRIII<sup>-/-</sup>) mice were purchased from Oriental BioService (Kyoto, Japan). BALB/c Fcε receptor Iα-deficient (FcεRIα<sup>-/-</sup>) mice were obtained from Jackson Laboratory (Bar Harbor, ME) and backcrossed for 11 generations into the C57BL/6 background. BALB/c ΔdblGATA mice were purchased from Jackson Laboratory (23). C57BL/6 FcRγ<sup>-/-</sup> mice were described previously (24). All mice were bred under specific-pathogen-free conditions at the animal facilities of Hyogo College of Medicine, Nishinomiya, Japan, and were used at 7 to 12 weeks of age. All animal experiments were conducted according to the guidelines for animal experiments at Hyogo College of Medicine.

**Antibodies.** Anti-IgE monoclonal antibody (MAb)-producing hybridoma 6HD5 was kindly provided by K. Okumura (Jundendo University, Tokyo, Japan) (25). Hybridoma JFP-1, producing an anti-green fluorescent protein MAb (RBRC-RCB2309), was provided by Riken BRC through the National Bio-Resource Project of the MEXT, Japan (26). MAbs were purified from ascites by affinity chromatography (HiTrap protein G HP; GE Healthcare, Little Chalfont, United Kingdom). Anti-2,4,6-trinitrophenyl (TNP) IgG1 (A111-3), anti-TNP IgE (C38-2), and biotinylated anti-IgE (R35-118) were purchased from BD Pharmingen (San Diego, CA). Anti-CD3 (2C-11), anti-CD28 (37.51), and anti-CD16/32 (93) were from Biolegend (San Diego, CA). Goat anti-IgG1, biotinylated goat anti-IgG1, and anti-IgE (23G3) were from Southern Biotech (Birmingham, AL).

**Parasitological techniques.** A strain of *Strongyloides venezuelensis* was previously described (27) and maintained by serial passage in Wistar rats. The third-stage infective larvae (L3) were obtained from fecal culture by the filter paper method. Mice were infected by subcutaneous (s.c.) inoculation with 3,000 to 4,000 L3. The degree of infection was monitored by

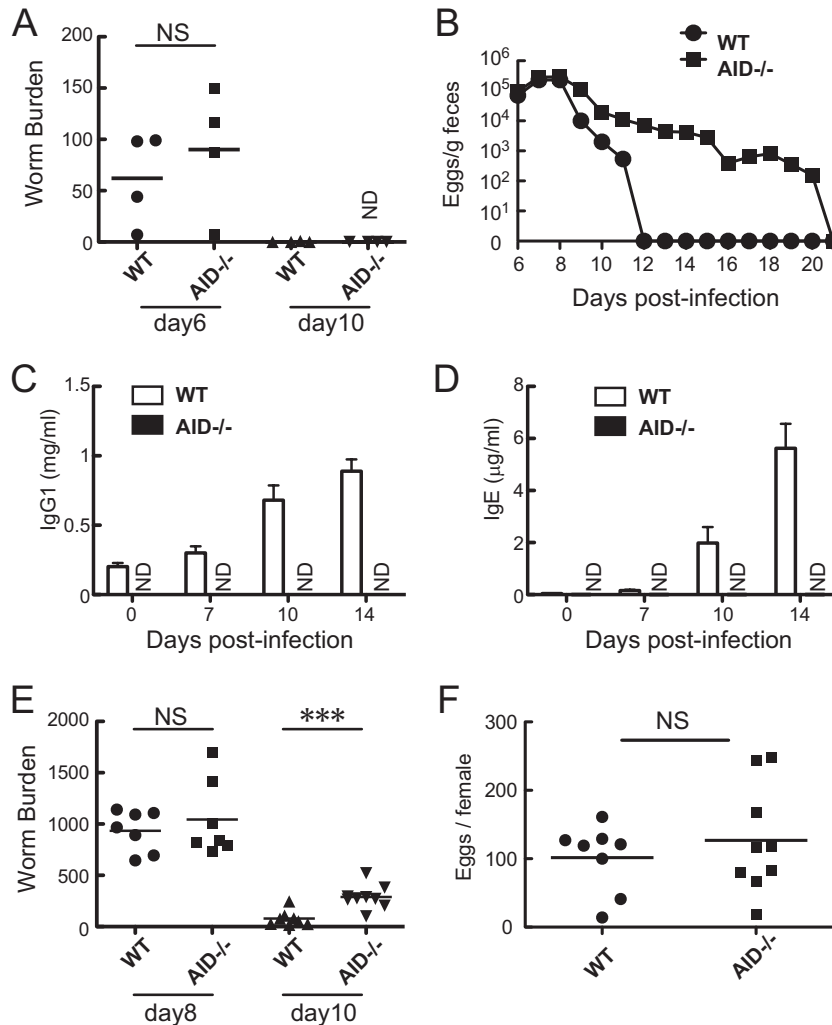
the number of eggs excreted per g feces or the recovery of adult worms from the small intestine. To obtain adult worms, the upper half of the small intestine from each infected mouse was removed, washed, cut open longitudinally, and incubated at 37°C in a 50-ml tube containing 30 ml of phosphate-buffered saline (PBS). Following 4 h of incubation, the tube was shaken vigorously, and worms that were shaken off the intestinal tissue were quantified under a microscope. Worm fecundity was calculated by dividing the number of eggs eliminated in feces by the number of worms recovered from the intestine of each mouse, as previously described (28). A strain of *Nippostrongylus brasiliensis* was maintained in our laboratory by serial passage in Sprague-Dawley rats. L3 were obtained from fecal culture by the filter paper method. Mice were infected by s.c. inoculation with 500 L3. To assess worm burdens, the whole small intestine from each infected mouse was removed and incubated on a steel mesh put on a beaker containing 100 ml PBS. Following 2 h at 37°C, worms that emerged from the intestinal tissue were quantified under a microscope.

**Generation of immune sera.** WT C57BL/6 mice were infected s.c. with 3,000 *S. venezuelensis* L3 and bled by heart puncture on day 14 postinfection (p.i.). Sera were separated by centrifugation and used as the primary immune (1°) sera. To boost Ab production, WT C57BL/6 mice and BALB/c ΔdblGATA mice were infected twice with 3,000 L3 at 30-day intervals. Sera were collected 7 to 9 days after the second infection and were designated secondary immune (2°) sera for WT C57BL/6 mouse-derived sera and hyperimmune (HI) sera for BALB/c ΔdblGATA mouse-derived sera. Nonimmune (0°) sera were obtained from naive C57BL/6 mice or BALB/c mice. IgG (SvIgG) from HI sera was separated by affinity chromatography (HiTrap protein G), and its flowthrough fraction was collected as IgG-depleted (ΔIgG) sera. More than 90% depletion of IgG was achieved in the process. IgE (SvIgE) was purified from ΔIgG sera by affinity chromatography using a 6HD5 MAb-coupled HiTrap NHS column (GE Healthcare), and its flowthrough fraction was collected as IgG- and IgE-depleted (ΔIgG ΔIgE) sera. More than 96% depletion of IgE was achieved in the process. To obtain IgE-inactivated (ΔIgE) sera, HI sera were heated to 56°C for 30 min. Purified IgG contained 0 to 0.5 μg/ml IgE. Purified IgE contained no detectable IgG. To obtain immune sera against *N. brasiliensis* (Nb sera), ΔdblGATA mice were infected twice with 500 *N. brasiliensis* L3 at 30-day intervals and bled from the heart on day 7 or 8 after the second infection.

**Measurement of IgG1, IgE, and total IgG.** Concentrations of IgG1 and IgE were measured by sandwich enzyme-linked immunosorbent assay (ELISA). The assay was performed on Costar 9018 96-well plates (Corning, New York, NY). The capture Abs (goat anti-mouse IgG1 for IgG1 and clone 23G3 for IgE) were used at a dilution of 1:200, and the biotinylated detection Abs (goat anti-mouse IgG1 for IgG1 and clone R35-118 for IgE) were used at 1:10,000. The signal was developed with TMB Microwell peroxidase substrate (KPL, Gaithersburg, MD), and the absorbance at 450 nm was recorded after H<sub>2</sub>SO<sub>4</sub> addition, using a Benchmark Plus microplate spectrophotometer (Bio-Rad, Hercules, CA). Anti-TNP IgG1 and anti-TNP IgE were used for standardization of IgG1 and IgE, respectively. Concentrations of total IgG were measured by use of a mouse IgG enzyme immunoassay (EIA) kit (TaKaRa Bio Inc., Otsu, Japan). Anti-TNP IgG1 was used for standardization of total IgG.

**T cell cytokine assay.** Mesenteric lymph nodes were harvested on day 10 p.i. with 4,000 L3, and CD4<sup>+</sup> T cells were isolated using magnetically activated cell sorting (MACS) beads (Miltenyi Biotec). Isolated CD4<sup>+</sup> T cells were resuspended in RPMI 1640 complete medium and stimulated on plates coated with 1 μg/ml anti-CD3 and 1 μg/ml anti-CD28. After 48 h of incubation, concentrations of IL-4 and gamma interferon (IFN-γ) in the culture supernatants were measured by DuoSet ELISA (R&D Systems).

**Cytokine assays *in vivo*.** To measure IL-4 secretion *in vivo*, mice were injected intraperitoneally (i.p.) with 10 μg biotinylated rat anti-mouse IL-4 Ab, and sera were collected 20 h later. The serum levels of IL-4 were measured by an *in vivo* capture assay set (BD Biosciences, San Diego, CA).



**FIG 1** *Nippostrongylus brasiliensis* and *Strongyloides venezuelensis* infections in wild-type (WT) and activation-induced cytidine deaminase-deficient ( $AID^{-/-}$ ) mice. (A) Numbers of worms recovered from small intestines on days 6 and 10 after infection with 500 L3 of *N. brasiliensis*. Note that one worm was collected from one WT mouse on day 10. NS, not significant; ND, not detected. Results are representative of two independent experiments and are presented as mean values for four mice per group. (B) Fecal egg excretion in WT and  $AID^{-/-}$  mice after infection with 4,000 L3 of *S. venezuelensis*. Results are representative of three independent experiments and are presented as mean values for four mice per group. (C and D) WT and  $AID^{-/-}$  mice were bled on days 0, 7, 10, and 14 after infection with 4,000 L3 of *S. venezuelensis*. Serum levels of IgG1 (C) and IgE (D) were determined by ELISA. Results are representative of two independent experiments and are presented as mean values and SEM for four mice per group. (E) Numbers of worms recovered from small intestines on days 8 and 10 after infection with 4,000 L3 of *S. venezuelensis*. (F) Fecundity of each female adult worm on day 10 after infection with 4,000 L3 of *S. venezuelensis*. In panels E and F, the results are representative of three independent experiments and are presented as mean values for seven to nine mice per group. \*\*\*,  $P < 0.001$  by Student's *t* test.

**Mucosal mast cell assessment.** Serum levels of mast cell protease 1 (MCPT-1) were measured with a mouse MCPT-1 ELISA kit (eBioscience, San Diego, CA). For histological examination of mucosal mast cells, a portion of the small intestine spanning 6 to 8 cm from the pyloric ring was removed, opened longitudinally, flattened on a filter paper, fixed in Carnoy's fluid, and stained with 0.1% alcian blue, pH 0.3. The number of mast cells in the epithelium and lamina propria under 10 villi was counted and presented per villous crypt unit (29).

**Fc $\gamma$ RII/III blocking and IgE neutralization *in vivo*.** To block Fc $\gamma$ RII/III *in vivo*, 50  $\mu$ g of anti-CD16/CD32 (93) was injected i.p. on days 6 and 8 p.i. with 4,000 L3. To neutralize IgE *in vivo*, 50  $\mu$ g of anti-IgE (6HD5) was injected i.p. on days 6 and 8 p.i. with 4,000 L3. Anti-green fluorescent protein MAb JFP-J1 (rat IgG2a) was used as an isotype control.

**Statistical analysis.** Statistical significance was examined by unpaired two-tailed Student's *t* test or one-way analysis of variance (ANOVA) fol-

lowed by Tukey's or Dunnett's *post hoc* test, as indicated in the text and the figure legends. Prism 5 (GraphPad, San Diego, CA) was used for data handling, analysis, and graphic representation. All significant comparisons are expressed as follows: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; and \*\*\*,  $P < 0.001$ .

## RESULTS

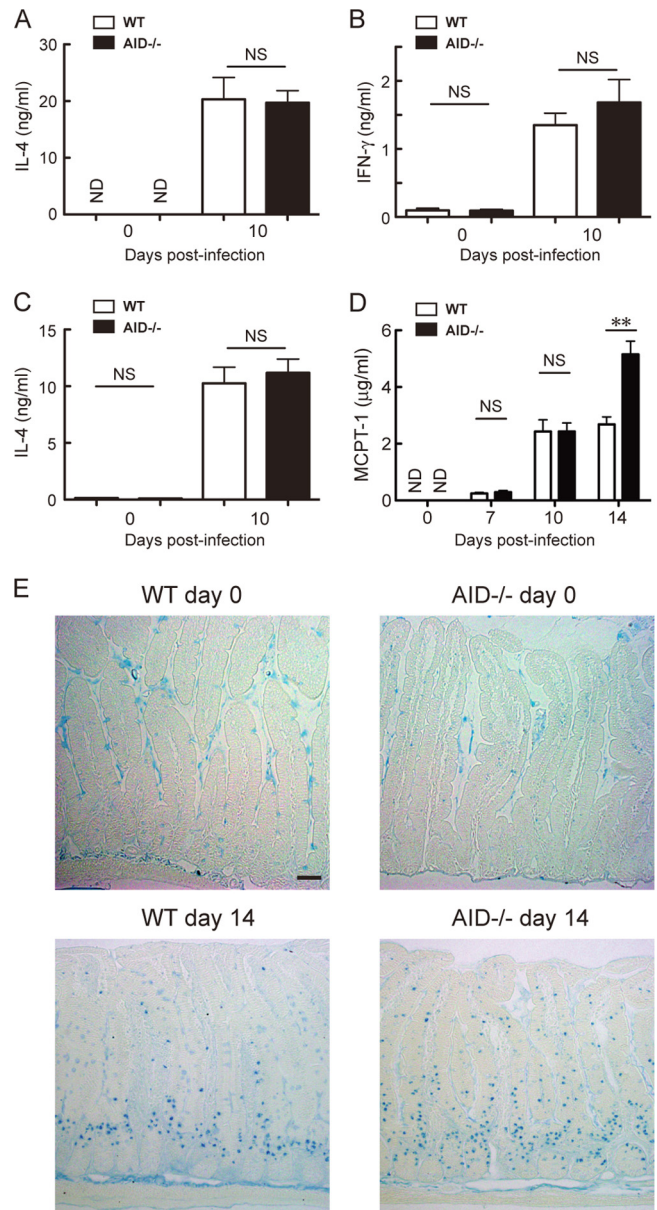
### *S. venezuelensis* expulsion was impaired in the absence of AID.

To examine the roles of Ab class switching during a primary infection with *N. brasiliensis* or *S. venezuelensis*, we took advantage of  $AID^{-/-}$  mice. We infected WT mice and  $AID^{-/-}$  mice with *N. brasiliensis* or *S. venezuelensis* L3 (Fig. 1A and B). Like WT mice,  $AID^{-/-}$  mice completed expulsion of *N. brasiliensis* by day 10 p.i. (Fig. 1A). In contrast to the complete expulsion of *S. venezuelensis* by day 12 seen in WT mice,  $AID^{-/-}$  mice required an additional 9

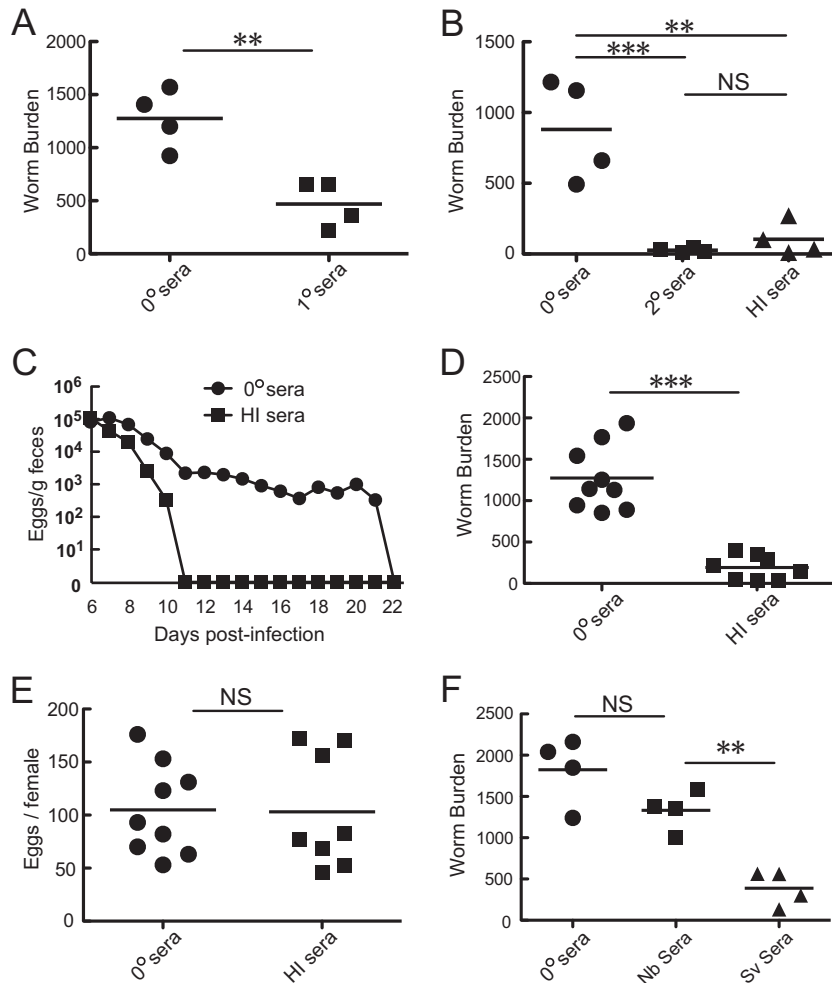
days to do so (Fig. 1B). These results revealed that  $AID^{-/-}$  mice exhibited impairment in expelling *S. venezuelensis* but showed no defect in expelling *N. brasiliensis*. We then asked what essential component is required in the host defense mechanisms against *S. venezuelensis*. We suspected that class-switched Abs play a protective role during infection with *S. venezuelensis*. Whereas WT mice showed strong induction of IgG1 and IgE following *S. venezuelensis* infection,  $AID^{-/-}$  mice were unable to produce IgG1 and IgE (Fig. 1C and D). Initial parasitization of adult worms in the small intestine was normal in  $AID^{-/-}$  mice, because adult worms recovered from small intestines of  $AID^{-/-}$  mice showed similar numbers to those from WT mice on day 8 p.i. (Fig. 1E). But eliminated eggs found in feces of  $AID^{-/-}$  mice outnumbered those for WT mice around day 9 or day 10 p.i. (Fig. 1B). This was coincident with increased numbers of adult worms recovered from  $AID^{-/-}$  mice compared to WT mice on day 10 p.i. (Fig. 1E) (on day 10, mean  $\pm$  standard error of the mean [SEM] of  $79 \pm 26$  worms [ $n = 8$ ] versus  $289 \pm 38$  worms [ $n = 9$ ];  $P = 0.0005$  by Student's *t* test). No differences were observed in the fecundity of adult worms hosted in WT mice and  $AID^{-/-}$  mice (Fig. 1F). Collectively, the class-switched Abs started working to expel *S. venezuelensis* around day 9, which coincided with the elevation of IgG1 and IgE.

**Th2-type immune responses and mucosal mast cell proliferation were intact in  $AID^{-/-}$  mice.** We assessed the potency of  $AID^{-/-}$  mice to provoke Th2-type immune responses following *S. venezuelensis* infection.  $CD4^+$  T cells were isolated from the mesenteric lymph nodes of WT mice and  $AID^{-/-}$  mice on day 10 p.i. and were stimulated with anti-CD3 and anti-CD28 *in vitro*.  $CD4^+$  T cells from  $AID^{-/-}$  mice produced levels of IL-4 and IFN- $\gamma$  similar to those from WT mice (Fig. 2A and B). IL-4 production *in vivo*, which was detected by an *in vivo* cytokine capture assay, did not differ between WT mice and  $AID^{-/-}$  mice on day 10 p.i. (Fig. 2C). We next examined the degree of intestinal mastocytosis in WT mice and  $AID^{-/-}$  mice after *S. venezuelensis* infection. We also examined the serum levels of MCPT-1, as serum levels of MCPT-1 are parallel with the activation and proliferation of mucosal mast cells. Before infection, the serum levels of MCPT-1 were not detected (Fig. 2D), and very few mucosal mast cells could be seen in both WT mice and  $AID^{-/-}$  mice (Fig. 2E, upper panels).  $AID^{-/-}$  mice produced comparable levels of MCPT-1 to those by WT mice on day 10 p.i. and produced twice as much as did WT mice on day 14 p.i. (Fig. 2D). The accumulations of mast cells in  $AID^{-/-}$  intestines also outnumbered those in WT controls on day 14 p.i. (Fig. 2E, lower panels) ( $33.3 \pm 2.7$ /villous crypt unit [ $n = 7$ ] versus  $16.8 \pm 1.4$ /villous crypt unit [ $n = 6$ ];  $P = 0.0004$  by Student's *t* test).  $AID^{-/-}$  mice had normal mast cell responses through day 10 but exacerbated mast cell responses on day 14 p.i., probably because the persistent infection continuously stimulated mast cell proliferation. These data showed that although Th2-type immune responses and mucosal mast cell proliferation occurred substantially in  $AID^{-/-}$  mice, worm expulsion was retarded compared to that in WT mice.

**Immune sera restored prompt worm expulsion in  $AID^{-/-}$  mice.** To investigate whether class-switched Abs can elicit *S. venezuelensis* adult worm expulsion, we passively transferred immune sera pooled from infected mice into  $AID^{-/-}$  mice (Fig. 3) and several lines of mutant mice (see Fig. 4 to 6) following infection with *S. venezuelensis*. To accomplish this aim, we determined the timing of serum transfer. Although Fig. 1 indicated that class-



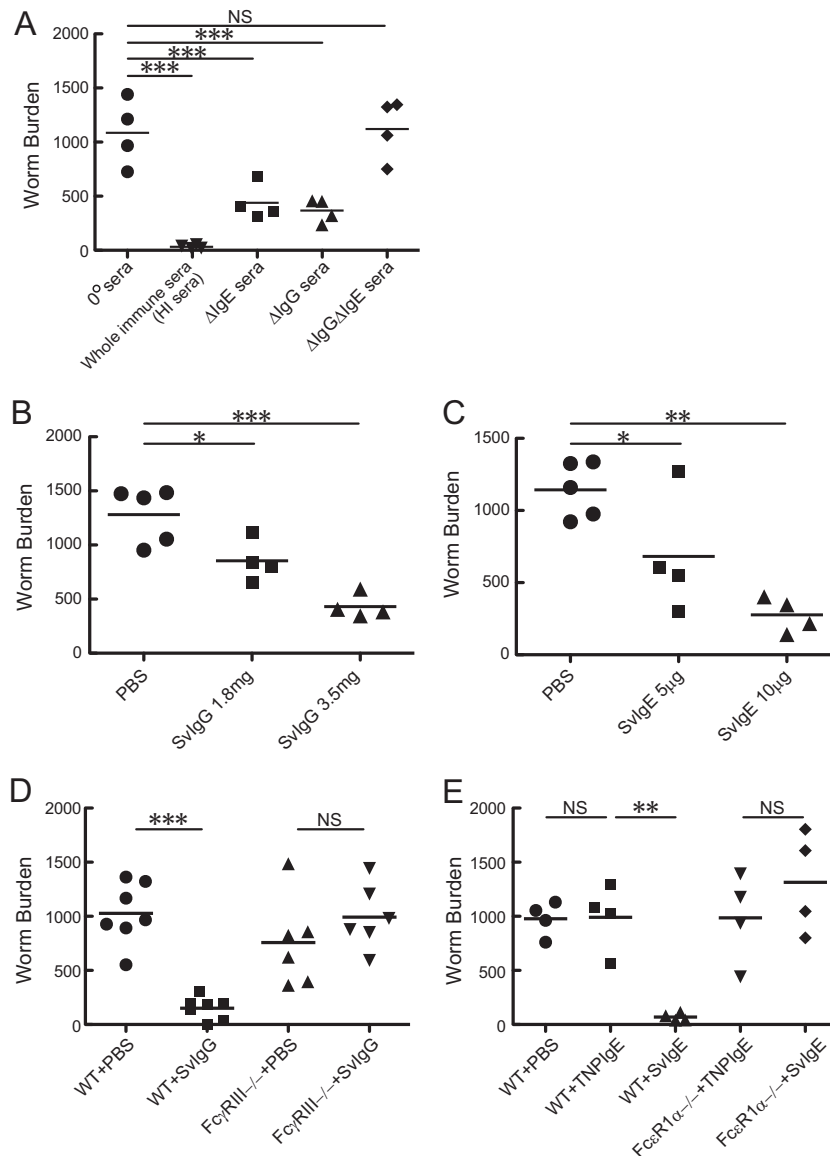
**FIG 2** T-helper 2-type immune responses and mucosal mast cell proliferation after *S. venezuelensis* infection. (A and B) WT or  $AID^{-/-}$  mice were infected with 4,000 L3 of *S. venezuelensis*.  $CD4^+$  T cells were isolated from the mesenteric lymph nodes on days 0 and 10 p.i. and were cultured on plates coated with 1  $\mu$ g/ml anti-CD3 and 1  $\mu$ g/ml anti-CD28 for 48 h. The concentrations of IL-4 and IFN- $\gamma$  in the supernatants were measured by ELISA. Results are representative of two independent experiments and are presented as mean values and SEM for four mice per group. (C) IL-4 secretion was evaluated by an *in vivo* cytokine capture assay on days 0 and 10 after infection with 4,000 L3. Results are representative of two independent experiments and are presented as mean values and SEM for four mice per group. (D) WT and  $AID^{-/-}$  mice were bled on days 0, 7, 10, and 14 after infection with 4,000 L3, and serum levels of MCPT-1 were measured by ELISA. Results are representative of two independent experiments and are presented as mean values and SEM for four to six mice per group. In panels A to D, statistical significance was evaluated by Student's *t* test. \*\*,  $P < 0.01$ . (E) On days 0 and 14 p.i., a portion of the small intestine was stained with alcian blue for visualization of mucosal mast cells. Similar results were obtained in two independent infections. Bar, 50  $\mu$ m.



**FIG 3** Effects of passively transferred immune sera on *S. venezuelensis* expulsion in  $AID^{-/-}$  mice. (A) Immune sera were collected from primarily infected WT C57BL/6 mice on day 14 after infection with 3,000 L3 of *S. venezuelensis* and were designated 1° sera. The IgE concentration of 1° sera was 3.5  $\mu\text{g/ml}$ .  $AID^{-/-}$  mice were injected i.p. with 1.5 ml of nonimmune sera (0° sera) or 1° sera on days 6 and 7, and adult worms were recovered from small intestines on day 8 after infection with 4,000 L3. Results are representative of two independent experiments and are presented as mean values for four mice per group. \*\*,  $P < 0.01$  by Student's *t* test. (B) Immune sera were collected from WT C57BL/6 mice and BALB/c  $\Delta\text{dblGATA}$  mice, both of which were infected twice with 3,000 L3 at 30-day intervals, and the sera were designated 2° sera and HI sera, respectively. IgE concentrations of 2° sera and HI sera were 5.0  $\mu\text{g/ml}$  and 50  $\mu\text{g/ml}$ , respectively.  $AID^{-/-}$  mice were injected i.p. with 1.0 ml of 0° sera, 1.0 ml of 2° sera, or 0.1 ml of HI sera on days 6 and 7, and adult worms were recovered from small intestines on day 8 after infection with 4,000 L3. Results are representative of two independent experiments and are presented as mean values for four mice per group. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  by one-way ANOVA with Tukey's *post hoc* test. (C) Four  $AID^{-/-}$  mice were injected i.p. with 0.1 ml of 0° sera or HI sera on days 6, 8, and 10 after infection with 4,000 L3, and eggs in feces were counted daily from day 6. Results are representative of two independent experiments and are presented as mean values for four mice per group. (D and E)  $AID^{-/-}$  mice were injected i.p. with 0.1 ml of 0° sera or HI sera on days 6 and 7, and adult worms were recovered from small intestines on day 8 after infection with 4,000 L3. Feces were collected on day 8, and the fecundity of each adult worm was calculated. Results are representative of two independent experiments and are presented as mean values for eight or nine mice per group. \*\*\*,  $P < 0.001$  by Student's *t* test. (F)  $AID^{-/-}$  mice were injected i.p. with 0.1 ml of 0° sera, *N. brasiliensis*-immune sera (Nb sera), or *S. venezuelensis*-immune sera (Sv sera) on days 6 and 7 after infection with 4,000 L3, and adult worms were recovered on day 8. Nb sera and Sv sera contained 140  $\mu\text{g/ml}$  of IgE and 50  $\mu\text{g/ml}$  of IgE, respectively. Results are representative of two independent experiments and are presented as mean values for four mice per group. \*\*,  $P < 0.01$  by one-way ANOVA with Tukey's *post hoc* test.

switched Abs started working around day 9 p.i., endogenously produced Abs would have compromised the effects of passively transferred immune sera in class switching-competent mice if serum transfer had started at day 9. Therefore, we transferred immune sera on days 6 and 7 repeatedly or on day 7 once. We then sacrificed mice on day 8 to examine the serum effect on worm burdens. Initial trials of injecting 0.2 ml of immune sera pooled from primarily infected WT C57BL/6 mice failed to reduce worm burdens due to the shortage of the injected volume (data not shown). However, repeated i.p. injections with 1.5 ml of immune

sera (1° sera) decreased worm burdens by more than 60% in  $AID^{-/-}$  mice (Fig. 3A). To reduce the injection volume, Ab production was boosted by secondary infection at 30-day intervals. Repeated i.p. injections with 1 ml of the boosted immune sera (2° sera) decreased worm burdens by more than 90% (Fig. 3B). However, repeated injections of 1 ml were excessive for 20- to 25-g mice and might have unfavorable influences on homeostasis. Moreover, 2-fold volume injection was required when IgG- and IgE-depleted sera were administered because fractionation steps diluted the original sera (Fig. 4A). Therefore, Ab-rich sera were



**FIG 4** Effects of fractionated immune sera on *S. venezuelensis* worm burdens. (A) Nonimmune sera ( $0^\circ$  sera; 0.2 ml), whole immune sera (HI sera; 0.2 ml), IgE-inactivated immune sera ( $\Delta$ IgE sera; 0.2 ml), IgG-depleted immune sera ( $\Delta$ IgG sera; 0.22 ml), and IgG- and IgE-depleted immune sera ( $\Delta$ IgG  $\Delta$ IgE sera; 0.4 ml) were injected intravenously (i.v.) into  $AID^{-/-}$  mice on days 6 and 7 after infection with 4,000 L3, and adult worms were recovered on day 8. Both 0.22 ml of  $\Delta$ IgG sera and 0.4 ml of  $\Delta$ IgG  $\Delta$ IgE sera originated from 0.2 ml of HI sera. HI sera contained 7.0 mg/ml of IgG and 50  $\mu$ g/ml of IgE. (B) PBS or HI serum-derived IgG (SvIgG) was injected i.v. into  $AID^{-/-}$  mice on day 7 after infection with 4,000 L3, and adult worms were recovered on day 8. A total of 3.5 mg of SvIgG originated from 0.5 ml of HI sera and contained 2.0 mg of IgG1. (C) PBS or HI serum-derived IgE (SvIgE) was injected i.v. into  $AID^{-/-}$  mice on day 7 after infection with 4,000 L3, and adult worms were recovered on day 8. Ten micrograms of SvIgE originated from 0.2 ml of HI sera. In panels A to C, results are representative of two independent experiments and are presented as mean values for four or five mice per group. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  by one-way ANOVA with Dunnett's *post hoc* test. (D) PBS or 1.8 mg of SvIgG was injected i.p. into WT mice or  $Fc\gamma RIII^{-/-}$  mice on days 6 and 7 after infection with 4,000 L3, and adult worms were recovered on day 8. (E) PBS, 5  $\mu$ g of anti-TNP IgE, or 5  $\mu$ g of SvIgE was injected i.p. into WT mice or  $Fc\epsilon R1\alpha^{-/-}$  mice on days 6 and 7 after infection with 4,000 L3, and adult worms were recovered on day 8. In panels D and E, results are representative of two independent experiments and are presented as mean values for four to seven mice per group. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  by one-way ANOVA with Tukey's *post hoc* test.

essential resources to avoid negative impacts on homeostasis potentially caused by volume overload. We empirically found that eosinophil-deficient BALB/c  $\Delta$ dblGATA mice showed a 3.4-fold increase in IgG1 production and 3.7-fold increase in IgE production relative to WT C57BL/6 mice (for IgG1, 3.48 mg/ml versus 1.02 mg/ml; and for IgE, 38.7  $\mu$ g/ml versus 10.5  $\mu$ g/ml [data are means for 5 pools of  $\Delta$ dblGATA mouse sera versus means for 2 pools of WT C57BL/6 mouse sera]).  $\Delta$ dblGATA mice showed

impairment in *S. venezuelensis* expulsion, which resulted in persistent infection like that in  $AID^{-/-}$  mice (our unpublished findings). Persistent infection provided the host sufficient time to produce effective Abs in both quantity and quality. Only 0.1 ml of the sera obtained from infected  $\Delta$ dblGATA mice showed comparable effects to 1 ml of the immune sera obtained from WT C57BL/6 mice (Fig. 3B). Therefore, we took advantage of these hyperimmune (HI) sera pooled from infected  $\Delta$ dblGATA mice in all sub-

sequent experiments. AID<sup>-/-</sup> mice transferred with the HI sera on days 6, 8, and 10 shortened the infection period from 22 days to 11 days (Fig. 3C). These effects were due to expulsion of adult worms from the small intestine but not to the suppression of fecundity (Fig. 3D and E). Next, we compared the activities of *S. venezuelensis*-immune sera (Sv sera) and *N. brasiliensis*-immune sera (Nb sera). Whereas Sv sera significantly reduced worm burdens, Nb sera exerted no notable effects on worm burdens (Fig. 3F). These data suggested that specific Abs against *S. venezuelensis* antigens played essential roles in expelling worms from the small intestine.

**Immune serum-derived IgG and IgE were able to induce worm expulsion.** Next, we examined which classes of Igs are able to induce worm expulsion. IgG depletion and IgE inactivation were achieved through affinity chromatography using protein G and through heat inactivation, respectively. Repeated intravenous injections of IgG-depleted ( $\Delta$ IgG) sera or IgE-inactivated ( $\Delta$ IgE) sera reduced worm burdens by about 60%, on average, compared to injections of nonimmune sera (0° sera) in *S. venezuelensis*-infected AID<sup>-/-</sup> mice (Fig. 4A). In contrast, whole immune sera (HI sera) decreased worm burdens by more than 90%, and the immune sera devoid of IgG and IgE ( $\Delta$ IgG  $\Delta$ IgE sera) showed no activities to promote worm expulsion (Fig. 4A). These results ruled out IgA as a relevant class of Ig in expelling worms and indicated that both IgG and IgE had the capability to exert *S. venezuelensis* expulsion. Furthermore, we fractionated IgG and IgE from immune sera (HI sera) to examine individual Ig activities. IgG fractionated from *S. venezuelensis*-immune sera (SvIgG) and IgE fractionated from *S. venezuelensis*-immune sera (SvIgE) induced worm expulsion in a dose-dependent manner (Fig. 4B and C). Additionally, we determined which Fc receptors play essential roles in IgG- and IgE-mediated worm expulsion. In mice, stimulatory IgG receptors are composed of Fc $\gamma$ RI, Fc $\gamma$ RIII, and Fc $\gamma$ RIV (19). By analogy to allergen-induced anaphylaxis (30), the low-affinity IgG receptor Fc $\gamma$ RIII is likely involved in IgG-mediated worm expulsion. SvIgG successfully induced worm expulsion in WT mice but not in Fc $\gamma$ RIII<sup>-/-</sup> mice (Fig. 4D). We then examined if IgE-mediated activity is exerted through the high-affinity IgE receptor (Fc $\epsilon$ RI). As expected, SvIgE significantly reduced worm burdens in WT mice but not in Fc $\epsilon$ RI $\alpha$ <sup>-/-</sup> mice (Fig. 4E). Collectively, the anti-*S. venezuelensis* activity found in the immune sera was carried out by IgG via Fc $\gamma$ RIII and by IgE via Fc $\epsilon$ RI.

**Collaboration between IgG and IgE in *S. venezuelensis* expulsion.** Passive transfer experiments indicated the requirement of IgG and IgE for *S. venezuelensis* expulsion via Fc $\gamma$ RIII and Fc $\epsilon$ RI, respectively. We examined whether Fc $\gamma$ RIII<sup>-/-</sup> mice or Fc $\epsilon$ RI $\alpha$ <sup>-/-</sup> mice exhibit retardation in *S. venezuelensis* expulsion compared to WT mice. In contrast to our expectation, both types of mutant mice showed no retardation in worm elimination (Fig. 5A and B). We speculated that IgE compensated the loss of IgG and vice versa during *S. venezuelensis* infection. This speculation proved to be correct, based on experiments using MAbs for IgE and Fc $\gamma$ R/III. IgE depletion by anti-IgE MAb treatment significantly retarded worm expulsion on day 10 p.i. in Fc $\gamma$ RIII<sup>-/-</sup> mice but not in WT mice (Fig. 5C). The worm burdens in IgE-depleted Fc $\gamma$ RIII<sup>-/-</sup> mice were comparable to those of Fc $\gamma$ <sup>-/-</sup> mice (Fig. 5C). Similar results were observed when IgG binding to Fc $\gamma$ RIII was inhibited by anti-Fc $\gamma$ R/III MAb treatment of Fc $\epsilon$ RI $\alpha$ <sup>-/-</sup> mice (Fig. 5D). Additionally, we examined collaborative effects between SvIgG and SvIgE in *S. venezuelensis* expulsion. While a

half-maximal-dose injection of SvIgG or SvIgE moderately accelerated worm expulsion, a combined administration of SvIgG and SvIgE significantly reduced worm burdens in *S. venezuelensis*-infected AID<sup>-/-</sup> mice (Fig. 5E). These data revealed that IgG and IgE played redundant roles but acted in concert to accelerate *S. venezuelensis* expulsion from infected animals.

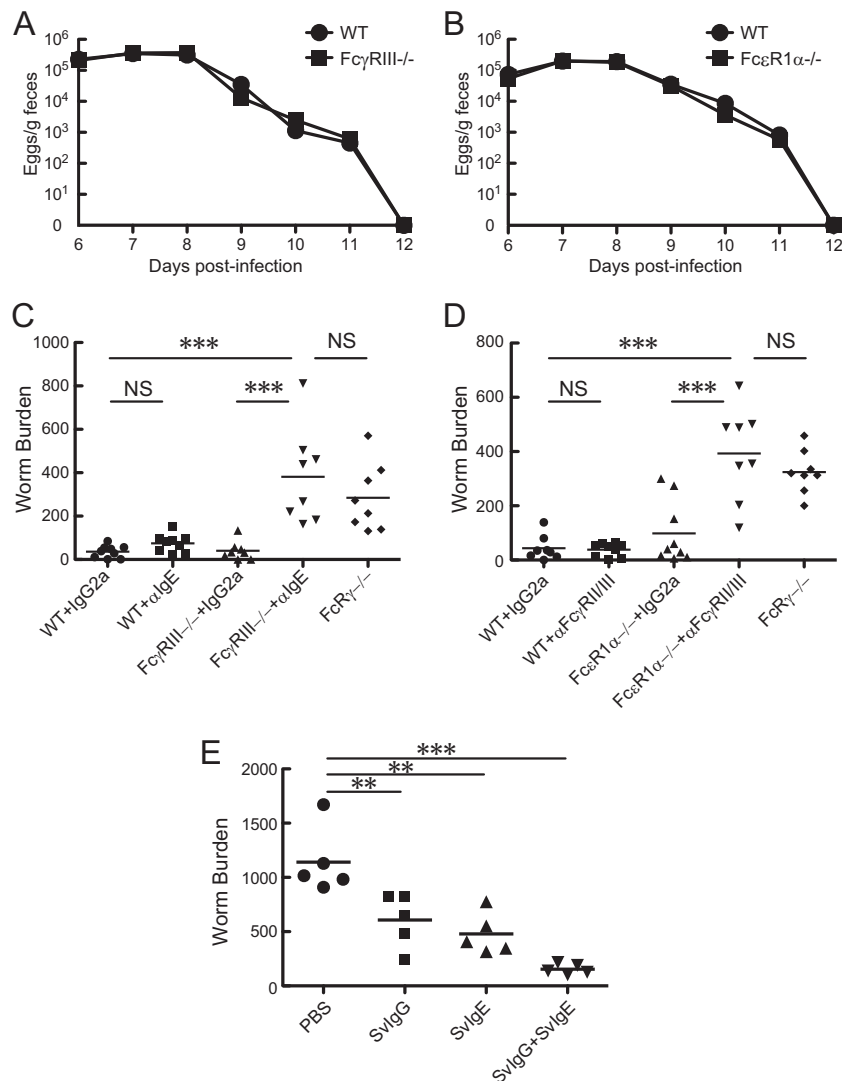
**Mast cells were indispensable for IgG- and IgE-mediated worm expulsion.** Previous studies demonstrated that mast cells play indispensable roles in *S. venezuelensis* expulsion from the small intestine (14–16) and that mast cells express Fc $\gamma$ RIII and Fc $\epsilon$ RI (31). This evidence led us to predict that mast cells are cellular targets of IgG and IgE for eliciting worm expulsion. As expected, repeated injections of SvIgG or SvIgE were not able to promote worm expulsion in mast cell-deficient WBB6F1-W/W<sup>y</sup> mice (Fig. 6A and B). Since adult worm numbers recovered from littermate WBB6F1<sup>+/+</sup> mice were significantly lower than those from WBB6F1-W/W<sup>y</sup> mice (99 ± 43 [n = 12] versus 1,470 ± 100 [n = 11]; P < 0.0001 by Student's *t* test) on day 8 p.i. under PBS-treated conditions, it was difficult to acquire reliable data from WBB6F1<sup>+/+</sup> mice. Thus, WT C57BL/6 mice were employed as controls instead of WBB6F1<sup>+/+</sup> mice (Fig. 6A and B). These data provided evidence that mast cells were indispensable for SvIgG- and SvIgE-mediated worm expulsion.

## DISCUSSION

*N. brasiliensis* and *S. venezuelensis* are two typical gastrointestinal nematodes hosted by rodents, and the mechanisms by which these nematodes are expelled have been topical themes in parasite immunology. IL-4/IL-13 effects, including excessive mucus production from intestinal goblet cells and enhanced contractility of smooth muscles in the small intestine, are essential for *N. brasiliensis* expulsion (1, 2). In contrast, the presence of B cells is dispensable for *N. brasiliensis* expulsion during primary and secondary infections (3). Our data also demonstrated that Ig class switching-deficient AID<sup>-/-</sup> mice normally expelled *N. brasiliensis* (Fig. 1F). These findings confirm the requirement of IL-4/IL-13 for expulsion of *N. brasiliensis* and formally rule out the contribution of class-switched Abs to this expulsion. IL-3/IL-9-induced intestinal mastocytosis plays a major role in expelling *S. venezuelensis* (14–16). However, a previous report (18) and our data in this study indicate that intestinal mastocytosis is insufficient for promptly eliminating *S. venezuelensis* in the absence of Fc $\gamma$  or AID.

We revealed that IgG and IgE accelerate *S. venezuelensis* expulsion and determined Fc receptors which play central roles in these processes. Immune serum-derived IgG and IgE induced worm expulsion via Fc $\gamma$ RIII and Fc $\epsilon$ RI, respectively (Fig. 4D and E). However, the kinetics of worm expulsion observed in Fc $\gamma$ RIII<sup>-/-</sup> mice or Fc $\epsilon$ RI $\alpha$ <sup>-/-</sup> mice were comparable in magnitude to those seen in WT mice (Fig. 5A and B). This discrepancy can be explained by a compensatory function exerted by IgE in the absence of IgG or by IgG in the absence of IgE. IgE neutralization in Fc $\gamma$ RIII<sup>-/-</sup> mice or Fc $\gamma$ RIII blockade in Fc $\epsilon$ RI $\alpha$ <sup>-/-</sup> mice retarded worm expulsion at a level equivalent to that in Fc $\gamma$ <sup>-/-</sup> mice (Fig. 5C and D). These data indicate that dual defects in signaling from Fc $\gamma$ RIII and Fc $\epsilon$ RI are responsible for the delayed worm expulsion observed in AID<sup>-/-</sup> mice as well as Fc $\gamma$ <sup>-/-</sup> mice.

Our findings also provide evidence that mast cells are indispensable for IgG- and IgE-mediated *S. venezuelensis* expulsion (Fig. 6A and B). Mast cells express Fc $\gamma$ RIII and Fc $\epsilon$ RI (31), both of



**FIG 5** Collaboration between IgG and IgE in *S. venezuelensis* expulsion. (A) Fecal egg excretion in WT and Fc $\gamma$ RIII $^{-/-}$  mice after infection with 4,000 L3. Results are representative of two independent experiments and are presented as mean values for five mice per group. (B) Fecal egg excretion in WT and Fc $\epsilon$ R1 $\alpha^{-/-}$  mice after infection with 4,000 L3. Results are representative of three independent experiments and are presented as mean values for five mice per group. (C) WT and Fc $\gamma$ RIII $^{-/-}$  mice were injected i.p. with 50  $\mu$ g of anti-IgE or an isotype control Ab on days 6 and 8 after infection with 4,000 L3. Fc $\gamma$ RIII $^{-/-}$  mice were infected with 4,000 L3 in parallel. Adult worms were recovered on day 10. In panels C and D, results are representative of combined data from two individual experiments and are presented as mean values for eight or nine mice per group. \*\*\*,  $P < 0.001$  by one-way ANOVA with Tukey's *post hoc* test. (E) PBS, 1.8 mg of SvlgG, 5  $\mu$ g of SvlgE, or a mixture of 1.8 mg of SvlgG and 5  $\mu$ g of SvlgE was injected i.v. into AID $^{-/-}$  mice on day 7 after infection with 4,000 L3, and adult worms were recovered on day 8. Results are representative of two independent experiments and are presented as mean values for five mice per group. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  by one-way ANOVA with Dunnett's *post hoc* test.

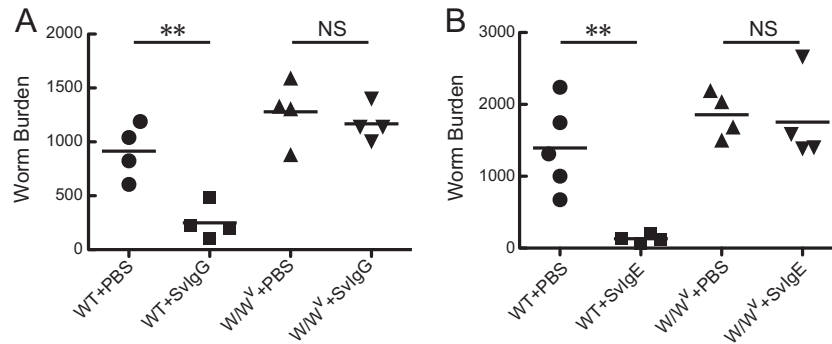
which employ Fc $\gamma$  to transduce signals from bound Igs, and mast cell-deficient mice were unable to expel *S. venezuelensis* in response to repeated administrations with immune serum-derived IgG or IgE (Fig. 6A and B). Collectively, these studies suggest that IgG and IgE act through Fc $\gamma$  to fully activate mast cells to accelerate *S. venezuelensis* expulsion.

In mice, IgGs consist of four different subclasses: IgG1, IgG2a/c, IgG2b, and IgG3. IgG1, IgG2a/c, and IgG2b are able to bind to Fc $\gamma$ RIII, but which subclasses dominate Fc $\gamma$ RIII activation depends on the model systems studied (19). Since *S. venezuelensis* infection accompanies IgG1 induction, like the case for other helminth infections (Fig. 1C), IgG1 is the plausible activator

of Fc $\gamma$ RIII. However, further fractionations from the immune sera are needed to clarify this prediction.

A passive transfer of immune sera before infection suppresses fecal larval outputs or adult worm recoveries of *S. ratti* (11, 12). Additionally, secondary infection studies indicate that larvae are destroyed before reaching the small intestine (27, 32). Many of the migratory larvae are surrounded by eosinophils, neutrophils, and mononuclear cells and are disintegrated in the lung (33). These studies indicate that immune sera disable the migratory larvae at an early stage during the course of infection. Furthermore, a series of studies analyzing *S. stercoralis* L3 contained under the mouse skin has demonstrated that complement C3, IgM, and IgG consist





**FIG 6** Requirement of mast cells for IgG- and IgE-mediated *S. venezuelensis* expulsion. (A) PBS or 1.8 mg of SvIgG was injected i.v. into WT C57BL/6 mice or WBB6F1-W/W<sup>v</sup> mice on days 6 and 7 after infection with 4,000 L3 of *S. venezuelensis*, and adult worms were recovered on day 8. (B) PBS or 5 μg of SvIgE was injected i.v. into WT C57BL/6 mice or WBB6F1-W/W<sup>v</sup> mice on days 6 and 7 after infection with 4,000 L3, and adult worms were recovered on day 8. \*\*,  $P < 0.01$  by one-way ANOVA with Tukey's *post hoc* test.

of essential factors in humoral immunity elicited by *S. stercoralis* L3 immunization (34–36). Collectively, immune sera exert high activities to eliminate larvae migrating from the skin to the lung in a challenge infection. However, it remained unsolved whether the administration of immune sera accelerates adult worm expulsion from the small intestine.

Murrell reported that immune sera did not induce expulsion of *S. ratti* adult worms from the small intestines of rats when they were administered between day 1 and day 5 after infection (12). Since the half-life of IgE is 12 h *in vivo* (37) and the mucosal mast cell accumulation is insufficient at those time points, the timing of transferring immune sera might have been premature to see the immune sera's effect on adult worm expulsion. In our protocol, immune sera or immune serum-derived fractions were administered to the recipient mice on days 6 and 7 or on day 7 only. On day 6, living larvae have already left the lung, reached the small intestine, and become adult worms (27). Furthermore, the absence of AID had no influence on the initial habitation of adult worms in the small intestine (Fig. 1E), because induction of IgG1 and IgE did not occur before the larvae reached the small intestine during primary infection (Fig. 1C and D). Therefore, our findings clearly demonstrate that immune serum-derived IgG and IgE accelerate *S. venezuelensis* expulsion when adult worms reside in the small intestine. Nevertheless, since the host species and *Strongyloides* species differ between Murrell's study and ours, a combination of these different features may result in contradictory results.

The advantage of concurrent production of IgG1 and IgE during helminth infections remains elusive. Serum concentrations of IgG1 and IgE were about 1 mg/ml and 0.005 mg/ml, respectively, on average, on day 14 p.i. for *S. venezuelensis* (Fig. 1C and D). The quantitative gap between high concentrations of IgG1 and low concentrations of IgE seems to reflect a low affinity of IgG1 for FcγRIII and a high affinity of IgE for FcεRI, respectively. A combination of moderate doses of IgG (1.8 mg) and IgE (5 μg) revealed a high capability of expelling worms at similar levels to those with a high dose of IgG (3.5 mg) or IgE (10 μg) (Fig. 4B and C and 5E). These data imply that large quantities of IgG are unnecessary when small quantities of IgE are concurrently produced during *S. venezuelensis* infection, because small quantities of IgE have potential equal to that of large quantities of IgG in driving *S. venezuelensis* expulsion.

A high dose of IgG alone has a high capability to expel *S. venezuelensis* (Fig. 4B). Thus, what is the advantage of IgE class switching during *S. venezuelensis* infection? There may be a cut in expenditure of the immune system benefited by the high capability of IgE. Secretion of large quantities of IgG requires expansion of Ig-producing cells, accelerated production of Ig per cell, or both. IgE class switching could decrease the requisite amounts of total Ig for eliciting worm expulsion, which would lighten the burden imposed on the host immune system. Another possibility is that the host can avoid extremely high production of IgG, which may lead to excessive formation of immune complexes. Animals infected with *S. venezuelensis* may regulate to moderately produce IgG, which in combination with IgE could fully activate mast cells for eliciting *S. venezuelensis* expulsion.

Given the anti-*Strongyloides* activities of IgG and IgE in mice, asymptomatic patients may inhibit worm dissemination from latently infected sites by the activities of these Abs. Thus, immunoglobulins prepared from people living in areas of endemicity may become an adjunct to existing therapy to prevent or cure hyperinfection. Our findings also provide implications in developing vaccines against *Strongyloides* species.

In summary, we clearly demonstrated that IgG and IgE exert collaborative effects on *S. venezuelensis* expulsion and act through FcγRIII and FcεRI, respectively. We also propose that mast cells are cellular targets of IgG and IgE in expelling the worms. However, we know little about the mechanisms by which the activated mast cells could exert their functions against *S. venezuelensis* adult worms in the small intestine. We need further studies to clarify the elaborate processes by which *S. venezuelensis* is expelled by the host immune system.

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