

IL-18 with IL-2 protects against *Strongyloides venezuelensis* infection by activating mucosal mast cell–dependent type 2 innate immunity

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C57BL/6 (B6) and B6 background STAT6^{-/-} mice pretreated with IL-18 plus IL-2 showed prominent intestinal mastocytosis and rapidly expelled implanted adult worms of the gastrointestinal nematode *Strongyloides venezuelensis*. In contrast, identically pretreated mast cell–deficient W/W^v mice failed to do so. Thus, activated mucosal mast cells (MMC) are crucial for parasite expulsion. B6 mice infected with *S. venezuelensis* third-stage larvae (L3) completed parasite expulsion by day 12 after infection, whereas IL-18^{-/-} or IL-18R α ^{-/-} B6 mice exhibited marked impairment in parasite expulsion, suggesting a substantial contribution of IL-18–dependent MMC activation to parasite expulsion. Compared with IL-18^{-/-} or IL-18R α ^{-/-} mice, *S. venezuelensis* L3–infected STAT6^{-/-} mice have poorly activated MMC and sustained infection; although their IL-18 production is normal. Neutralization of IL-18 and IL-2 further reduces expulsion in infected STAT6^{-/-} mice. These results suggest that collaboration between IL-18–dependent and Th2 cell–dependent mastocytosis is important for prompt parasite expulsion.

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Abbreviations used: MMC, mucosal mast cell; mMCP-1, mouse mast cell protease-1; L3, third-stage larvae; VCU, villous crypt units.

Microbes can be classified into intracellular and extracellular types. In general, intracellular microbes are expelled by cell-mediated immunity (Th1 responses), whereas extracellular microbe eradication often is mediated by the function of humoral immunity (Th2 responses; references 1–6). Upon infection with intracellular microbes, macrophages or DCs produce various types of proinflammatory cytokines in response to Toll-like-receptor/MyD88-mediated signaling (7, 8). Among the proinflammatory cytokines produced, IL-12 and IL-18 are most important up-stream cytokines of IFN- γ and synergistically induce T cells, B cells, NK cells, macrophages, and DCs to produce IFN- γ (9–16). Resultant IFN- γ then activates macrophages to produce nitric oxide, leading to eradication of intracellular pathogens (3–5). Indeed, IL-12– and/or IL-18–deficient mice show markedly reduced host resistance against *Cryptococcus neoformans* or *Leishmania major* (17–19). Thus, both IL-12 and IL-18 are important for host defense against intracellular microbes. However, our recent studies clarified that IL-18 without help from IL-12 induces Th2 cytokines in T cells,

basophils, and mast cells (4, 20–23). Most surprisingly, administration of IL-18 or IL-18 plus IL-2 into naive mice induces IgE in a CD4⁺ T cell–, IL-4–, and STAT6–dependent manner (20–22). Moreover, transgenic mice overexpressing IL-18 in their keratinocytes spontaneously produce IgE (21, 24) and develop atopic dermatitis (25). Thus, IL-18 regulates both Th1 and Th2 responses depending on its cytokine milieu (4).

It is well known that expulsion of some types of helminthes depends on the action of activated mast cells (2). Here we demonstrate that treatment of mice with daily injection of IL-2 plus IL-18 induces significant increases in the number of intestinal mucosal mast cells (MMCs) and in their release of mouse mast cell protease-1 (mMCP-1), which are hallmarks of infection with gastrointestinal nematode (2, 26–29). Furthermore, this pretreatment prepares them to expel implanted adult worms of *Strongyloides venezuelensis* rapidly. In contrast, identically pretreated W/W^v mice that lack mast cells (30) failed to expel implanted worms. These results suggest that IL-18– and

IL-2-dependent MMC activation is indispensable for rapid parasite expulsion. WT mice inoculated with *S. venezuelensis* third-stage larvae (L3) showed significant increases both in serum IL-18 and mMCP-1 levels and completed worm expulsion by 12 d. In contrast, IL-18-deficient (IL-18^{-/-}) or IL-18R α ^{-/-} mice required longer period to complete worm expulsion. STAT6^{-/-} mice infected with *S. venezuelensis* L3 showed more severe impairment in parasite expulsion, and neutralization of IL-18 and IL-2 further reduced their capacity to expel the parasite. Here, we demonstrate that both IL-18-dependent and Th2 cytokine-dependent MMC activation pathways are critically involved in induction of rapid parasite expulsion.

RESULTS

Intestinal MMC accumulation in WT mice injected with IL-18 plus IL-2

We first tested whether daily i.p. injection of IL-2 and/or IL-18 induces accumulation of MMC in intestines of the mice. Stained jejunal sections revealed that administration of IL-18 (2 μ g/d) and IL-2 (2,000 U/d) induced MMC, although treatment with each component alone did not induce or induced it weakly (Fig. 1 A). Titration study indicated that IL-18 stimulated a dose-dependent increase in MMC when combined with IL-2 (2,000 U/d) (Fig. 1 B).

We injected 2,000 U/d of IL-2. Much higher doses of IL-2 (e.g., 10⁴ U/d) failed to enhance this response (unpublished data). We compared the degree of accumulation of MMC in the mice treated with IL-2 and IL-18 with that in mice inoculated with *S. venezuelensis* L3 2 wk earlier and found that infection with *S. venezuelensis* L3 showed more potent MMC-inducing activity (Fig. 1 A, d and f, and B). The effect of IL-2 and IL-18 was prominent in intestines, and no mast cell accumulation was observed in other organs, such as lungs, spleens, livers, and kidneys.

Because IL-3, IL-4, and IL-9 are well-known potent mast cell growth factors (30–32), we simultaneously measured serum levels of these cytokines at various time points after treatment of mice with IL-2 plus IL-18. As shown in Fig. 1 C, administration of IL-18 (2 μ g/d) and IL-2 (2,000 U/d) induced increases in serum levels of IL-3, IL-4, IL-9, and IL-13. In general, these cytokines are below the detection level in normal mice. However, serum levels of IL-3, IL-4, and IL-9 increased in mice injected with IL-2 and IL-18 and peaked at d 7; IL-13 was persistently elevated even beyond d 7. IL-18 treatment alone induced IL-4 very modestly (30 pg/ml). Neither IL-10 nor IFN- γ was detected in the sera of mice injected with IL-18 plus IL-2 (unpublished data).

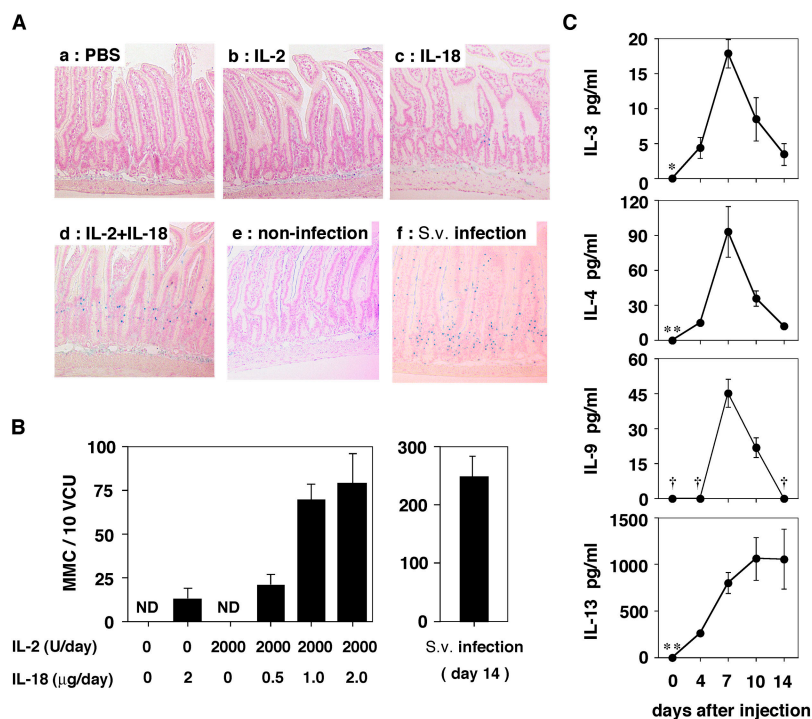


Figure 1. IL-18 plus IL-2-induced intestinal MMC accumulation in vivo. (A) C57BL/6 mice (six to eight mice per group) were injected daily i.p. with PBS alone or with IL-18 (0.5–2 μ g/d) and/or IL-2 (2,000 U/d) for 13 d or were inoculated with 5,000 *S. venezuelensis* L3. 14 d after cytokine treatments or inoculation with *S. venezuelensis* L3, intestine tissue samples (8–10 cm from the pyloric ring) were removed and fixed in Carnoy's fluid and stained with Alcian blue, pH 0.3, and Safranin-O. Original magnification, 200. (B) The

number of mast cells in the epithelium and lamina propria mucosa was counted in 10 VCU. Results are geometric means \pm SD. ND; not detected. (C) C57BL/6 mice (six to eight mice per group) were injected daily with IL-18 (2 μ g/d) plus IL-2 (2,000 U/d) for 13 d. They were bled 0, 4, 7, 10, and 14 d later, and serum IL-3, IL-4, IL-9, and IL-13 levels were measured by ELISA. Results are geometric means \pm SEM. *, <3 pg/ml; **, <10 pg/ml; †, <20 pg/ml. Results are representative of three independent experiments.

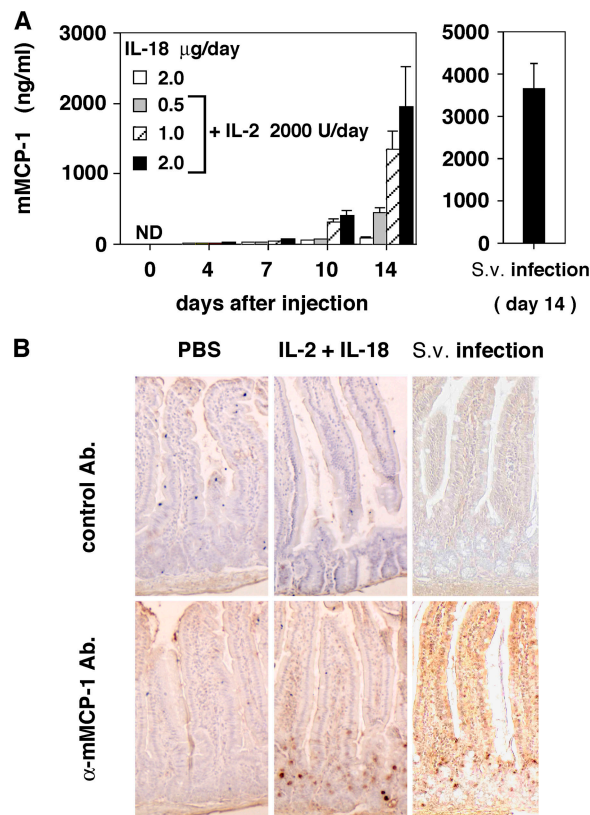


Figure 2. IL-18- plus IL-2-induced mMCP-1 production in vivo. (A) C57BL/6 mice (six to eight mice per group) were injected i.p. daily with IL-18 (2 μg/d) or with various doses of IL-18 (0.5–2 μg/d) and IL-2 (2,000 U/d) for 13 d or were inoculated with 5,000 *S. venezuelensis* L3. They were bled 0, 4, 7, 10, and 14 d later, and mMCP-1 in sera was measured by ELISA. Results are geometric means ± SEM. ND; not detected. (B) C57BL/6 mice (six to eight mice per group) were injected daily with PBS or with IL-18 (2 μg/d) and IL-2 (2,000 U/d) for 13 d or were inoculated with 5,000 *S. venezuelensis* L3. 14 d after treatments, upper intestines were removed and fixed with 4% paraformaldehyde. Immunohistochemical staining for mMCP-1 was performed as described in Materials and methods. Results are representative of three independent experiments.

mMCP-1 production by activated intestinal MMC in mice injected with IL-18 plus IL-2

It is well known that the serum level of mMCP-1 correlates very well with the degree of intestinal MMC activation that is associated with mucosal mast cell degranulation (26–29). Thus, we examined whether accumulated MMCs were activated to produce mMCP-1 in mice treated with IL-2 plus IL-18. As shown in Fig. 2 A, the serum level of mMCP-1 was below the detection level under normal conditions. However, when mice received a daily injection of IL-18 (2 μg/d), the mMCP-1 level in their sera increased (day 7, 19.5 ng/ml; day 10, 39.58 ng/ml; day 14, 55.6 ng/ml). Coinjection of IL-2 markedly increased these serum mMCP-1 levels. IL-18 induced a dose-dependent increase in mMCP-1 levels in mice treated with IL-2 (2,000 U/d; Fig. 2 A). We found that serum level of mMCP-1 in the mice injected with IL-18 (2 μg/d)

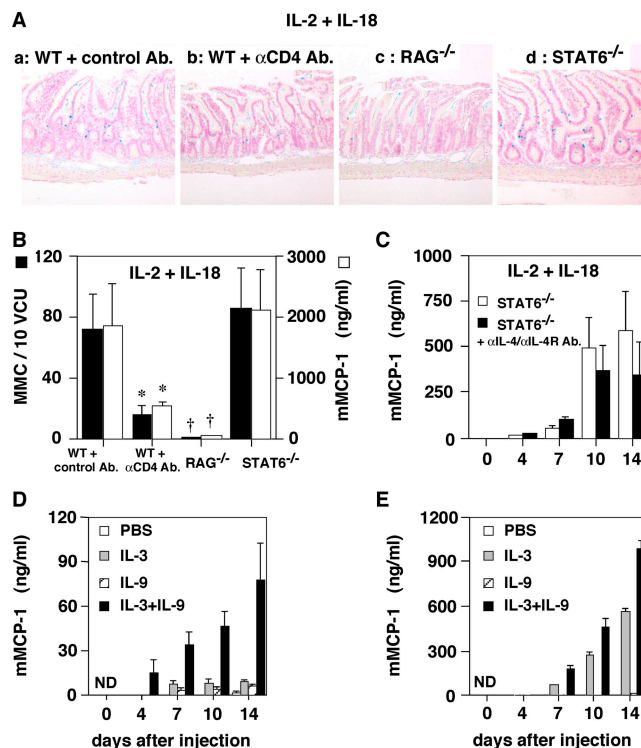


Figure 3. IL-18- plus IL-2-induced CD4⁺ T cell-dependent but STAT6-independent intestinal mastocytosis. C57BL/6 WT and C57BL/6-background RAG-2^{-/-} (RAG^{-/-}) or STAT6^{-/-} mice (six mice per group) were injected i.p. daily with IL-18 (2 μg/d) and/or IL-2 (2,000 U/d) for 13 d. To deplete CD4⁺ T cells, C57BL/6 mice received anti-CD4 (GK1.5; 0.5 mg/d) or control antibody (rat IgG2b; 0.5 mg/d) 7 and 4 d before IL-2 and IL-18 treatment and 0, 3, and 7 d after treatment. (A) 14 d after IL-2 and IL-18 treatment, tissue samples (8–10 cm) from the pyloric ring were removed and fixed in Carnoy's fluid and stained with Alcian blue, pH 0.3, and Safranin-O. Original magnification, 200. (B) The number of mast cells in the epithelium and lamina propria mucosa was counted in 10 VCU. Results are geometric means ± SD. Mice were bled 0, 7, 10, and 14 d later, and serum mMCP-1 was measured by ELISA. Results are geometric means ± SEM. *, P < 0.01; †, P < 0.001 by Student's t test as compared with mice treated with control antibody. Results are representative of three independent experiments. (C) STAT6^{-/-} mice (eight mice per group) were injected i.p. daily with IL-18 (2 μg/d) and/or IL-2 (2,000 U/d) for 13 d. Four mice from each group received a mixture of anti-IL-4 antibody (10 mg/d) and anti-IL-4R antibody (10 mg/d) 1 d before IL-2 and IL-18 treatment and 4 and 8 d after treatment. They were bled 0, 4, 7, 10, and 14 d later, and serum mMCP-1 was measured by ELISA. Results are geometric means ± SEM. Results are representative of two independent experiments. (D and E) C57BL/6 normal mice (five mice per group) were injected daily for 13 d (D) with PBS alone or with IL-3 (0.013 μg/d) and/or IL-9 (0.5 μg/d) or (E) with PBS alone or with IL-3 (0.1 μg/d) and/or IL-9 (0.5 μg/d). They were bled 0, 4, 7, 10, and 14 d later, and serum mMCP-1 levels were measured by ELISA. Results are geometric means ± SEM. ND; not detected. Results are representative of three independent experiments.

and IL-2 (2,000 U/d) was almost comparable with that in the mice inoculated with *S. venezuelensis* L3 (Fig. 2 A).

In addition to the effect of IL-18 and IL-2 on serum mMCP-1 level, stained jejunal sections indicated that this

treatment markedly increased the number of mMCP-1-positive cells (Fig. 2 B). Their distribution is very similar to that of MMC (Fig. 1 A and Fig. 2 B). Furthermore, like MMC, mMCP-1-positive cells were prominent in intestines and were not observed in the lungs, spleens, livers, and kidneys of the mice injected with IL-18 and IL-2 (unpublished data). Taken together, these results clearly indicated that treatment with IL-18 and IL-2 induces accumulation, maturation, and activation of intestinal MMC, namely intestinal mastocytosis.

CD4⁺ T cell-dependent but STAT6-independent mastocytosis in mice injected with IL-18 plus IL-2

To determine whether this intestinal mastocytosis induced by IL-18 plus IL-2 is dependent on the function of CD4⁺ T cells, we injected IL-2 and IL-18 into C57BL/6 WT mice depleted of CD4⁺ T cells by pretreatment with anti-CD4 antibody or into C57BL/6 RAG-2-deficient (RAG-2^{-/-}) mice lacking both T cells and B cells. Anti-CD4 treatment significantly impaired IL-2- and IL-18-induced intestinal mastocytosis (Fig. 3, A and B) and mMCP-1 production ($P < 0.01$; Fig. 3 B). Furthermore, RAG-2^{-/-} mice injected with IL-18 plus IL-2 showed very poor accumulation of MMC and very low serum levels of mMCP-1 (Fig. 3, A and B). These results clearly indicated that IL-18- plus IL-2-induced accumulation of intestinal MMC is dependent on the function of CD4⁺ T cells.

Injection of IL-2 and IL-18 induced increases in the serum levels of IL-3, IL-4, IL-9, and IL-13 (Fig. 1 C). We previously reported that IL-2 and IL-18 stimulate CD4⁺ T cells from C57BL/6 or C57BL/6 background STAT6^{-/-} mice to produce IL-3, IL-4, IL-9, and IL-13 (21, 22). Because IL-4 was shown to induce intestinal mastocytosis in a STAT6-independent manner (33, 34), we injected IL-2 and IL-18 into STAT6^{-/-} mice and examined the accumulation of MMC. STAT6^{-/-} mice displayed intestinal mastocytosis and high serum levels of mMCP-1 (Fig. 3, A and B) when injected with IL-2 and IL-18, suggesting STAT6-independent IL-4 induction of mastocytosis. However, blockage of IL-4 only partly inhibited the IL-2-plus IL-18-induced mMCP-1 response (Fig. 3 C). Thus, the effect of IL-4 was modest, and induction of mMCP-1 requires the participation of other factors. Because IL-3 and IL-9 can induce MMC in vivo (30–32), we daily injected IL-3 (0.013 $\mu\text{g}/\text{d}$) and IL-9 (0.5 $\mu\text{g}/\text{d}$) into C57BL/6 WT mice. This treatment only partially replaced the effect of IL-2 and IL-18 (Fig. 3 D). However, a higher dose of IL-3 (0.1 $\mu\text{g}/\text{d}$) even without IL-9 strongly increased the serum level of mMCP-1, and additional IL-9 stimulation enhanced this effect modestly (Fig. 3 E). Thus, IL-2 plus IL-18 treatment seems to induce mMCP-1 by virtue of IL-3 and IL-9 from CD4⁺ T cells, and IL-3 seems to be most critical for mMCP-1 induction.

Rapid expulsion of implanted adult *S. venezuelensis* by mice treated with IL-18 plus IL-2

Because the degree of parasite expulsion is closely associated with the degree of intestinal mastocytosis in the mice in-

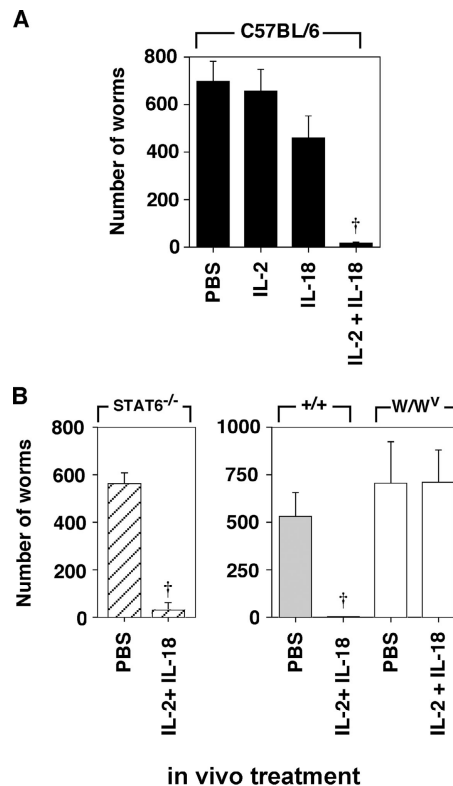


Figure 4. IL-18- plus IL-2-induced MMC-dependent expulsion of adult *S. venezuelensis*. Adult *S. venezuelensis* (1,500 worms/mouse) were implanted in the duodenum of recipient (A) C57BL/6 WT mice and (B) C57BL/6-background STAT6^{-/-}, WBB6F1^{+/+}, and WBB6F1-W/W^v mice that had been injected i.p. daily with PBS alone or with IL-18 (2 $\mu\text{g}/\text{d}$) and/or IL-2 (2,000 U/d) for 13 d. 16 h after implantation, the mouse intestines were removed, cut open longitudinally, and washed lightly to remove fecal matter and worms that were still in the lumen. Then the intestines were incubated in PBS at 37°C for 3 h. The number of worms that emerged from the intestines was counted. Results are geometric means \pm SD of eight animals per group and are representative of more than three independent experiments. †, $P < 0.0001$ by Student's *t* test as compared with PBS control groups.

ected with *S. venezuelensis* (35, 36), we examined the protective role of IL-2- and IL-18-induced MMC against *S. venezuelensis* infection. Thus, we implanted adult worms in the duodenum of mice pretreated with IL-2 and/or IL-18 for 13 d. We recovered invading parasites at 16 h after implantation (Fig. 4 A; reference 36). A considerable number of the parasites were shown to invade intestines of the mice that received PBS, IL-2, or IL-18 alone. Furthermore, these mice remained heavily infected up to 7 d after treatment (unpublished data). In contrast, mice pretreated with IL-18 plus IL-2 almost completely rejected them ($P < 0.0001$; Fig. 4 A), revealing this expulsion was a rapid response. In these experiments, we injected 2 $\mu\text{g}/\text{d}$ of IL-18 together with IL-2 into naive mice. However, we found that a much lower dose of IL-18 (0.5 $\mu\text{g}/\text{d}$) equally prepared mice to expel implanted parasites rapidly (unpublished data). It is important to start injection of a mixture of IL-2 and IL-18 before implan-

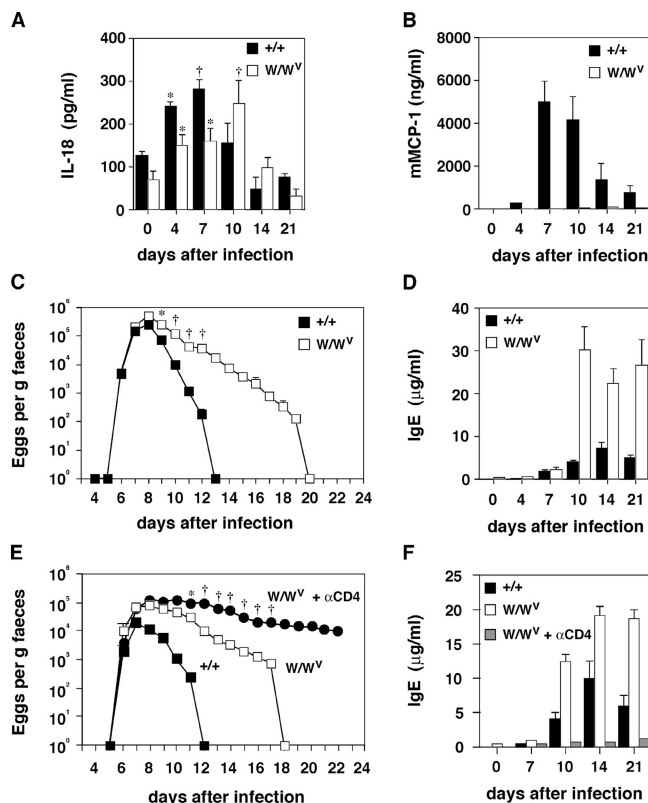


Figure 5. Role of MMC against *S. venezuelensis* L3. (A and B) Serum levels of (A) IL-18 and (B) mMCP-1 from WBB6F1^{+/+} and WBB6F1-W/W^v mice inoculated with 5,000 *S. venezuelensis* L3. Results are geometric means \pm SEM of five animals per group and are representative of three independent experiments. *, $P < 0.01$; †, $P < 0.001$ by Student's *t* test as compared with day 0. (C) Kinetics of the number of eggs per g feces and (D) serum levels of IgE from WBB6F1^{+/+} and WBB6F1-W/W^v mice inoculated with 5,000 *S. venezuelensis* L3. Results are geometric means \pm SEM of five animals per group. *, $P < 0.01$; †, $P < 0.005$ versus corresponding value for WBB6F1^{+/+} mice. (E) Kinetics of the number of eggs per g feces and (F) serum levels of IgE from WBB6F1^{+/+} mice, WBB6F1-W/W^v mice, and CD4⁺ T cell-depleted WBB6F1-W/W^v mice inoculated with 5,000 *S. venezuelensis* L3. To deplete CD4⁺ T cells, WBB6F1-W/W^v mice were injected with anti-CD4 antibody (0.5 mg/d) on days 7 and 4 before infection and two times per wk after infection. Results are geometric means \pm SEM of five animals per group. *, $P < 0.01$; †, $P < 0.005$ versus corresponding value for WBB6F1-W/W^v mice without anti-CD4 antibody treatment.

tation (unpublished data), because appreciable induction of MMC required >10 d (Fig. 2 A). These results clearly indicated that only mice pretreated with IL-2 plus IL-18 gained the capacity to expel implanted adult parasites rapidly. As expected from the results shown in Fig. 3, STAT6^{-/-} mice pretreated with IL-2 and IL-18 also gained the capacity to reject implanted parasites rapidly (Fig. 4 B).

Next, we examined whether mastocytosis induced by IL-2 plus IL-18 is critically involved in the expulsion of implanted parasites. Thus, we treated mast cell-deficient W/W^v mice with IL-2 and IL-18 and examined their capacity to

expel parasites. We found these mice failed to respond to this treatment by intestinal MMC accumulation and mMCP-1 production (unpublished data). Furthermore, they could not reject implanted parasites (Fig. 4 B). These results clearly indicated that rapid expulsion of implanted adult worms is mediated by the function of activated MMC.

Partial contribution of activated MMC to expulsion of *S. venezuelensis* L3

In the preceding section, we demonstrated that MMC activation is indispensable for rapid expulsion of implanted adult worms. Next, it was important to determine whether MMCs are also critically involved in host defense against *S. venezuelensis* L3 infection. Thus, we inoculated control mice and mast cell-deficient W/W^v mice (37) with 5,000 *S. venezuelensis* L3. We daily counted fecal egg number (eggs/g of feces). We simultaneously tested whether this *S. venezuelensis* L3 infection induced IL-18 production and found that both types of mice clearly produced IL-18 (Fig. 5 A). However, only control mice, but not W/W^v mice, produced mMCP-1 (Fig. 5 B). Thus, MMCs are the only cell source of mMCP-1 during *S. venezuelensis* L3 infection. Inoculated control mice completed parasite expulsion by day 13, whereas production of the parasites' eggs was significantly prolonged in W/W^v mice (Fig. 5 C). These results suggest that activated MMC markedly shortened the time required for parasite expulsion. However, we should be cautious in concluding that delayed worm expulsion by W/W^v mice reflects only a mast cell defect. These mice have defects in peristalsis (38) and intestinal T cell populations (39), as well as a mast cell defect. Despite these defects, W/W^v mice eventually expelled parasites by day 20 (Fig. 5 C), suggesting contribution of MMC-independent parasite expulsion mechanism. Indeed, W/W^v mice infected by *S. venezuelensis* showed a markedly augmented IgE response (Fig. 5 D), and depletion of CD4⁺ T cells abrogated their capacity to expel parasite and to produce IgE (Fig. 5, E and F). Thus, Th2 cells also play a protective role for parasite expulsion.

Impaired expulsion of *S. venezuelensis* L3 without help from endogenous IL-18

To address the role of endogenous IL-18 in the host defense against *S. venezuelensis* L3 infection, the capacity of IL-18^{-/-} mice and IL-18R α ^{-/-} mice to expel *S. venezuelensis* was examined. C57BL/6 WT, C57BL/6 background IL-18^{-/-} and IL-18R α ^{-/-} mice were inoculated with 5,000 *S. venezuelensis* L3. As shown in Fig. 6 A, IL-18^{-/-} and IL-18R α ^{-/-} mice, compared with infected WT mice, exhibited significantly prolonged production of the parasites' eggs, but they could expel worms by day 16. We found these deficient mice also exhibited significantly reduced levels of mMCP-1 at day 4, 7, and 10 after infection (Fig. 6 B), suggesting the importance of endogenous IL-18 for early induction of mMCP-1. However, these deficient mice and WT mice

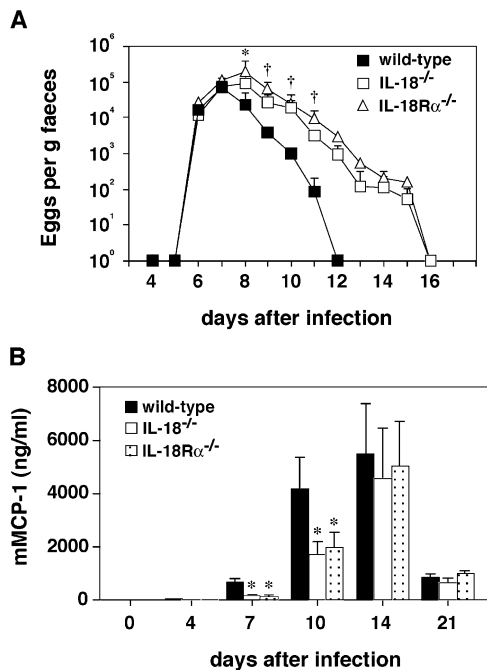


Figure 6. Role of endogenous IL-18 against *S. venezuelensis* L3. Kinetics of (A) the number of eggs per g feces and (B) serum levels of mMCP-1 from WT, IL-18^{-/-}, and IL-18Rα^{-/-} mice inoculated with 5,000 *S. venezuelensis* L3. Results are geometric means ± SEM of eight animals per group and are representative of three independent experiments. *, P < 0.01; †, P < 0.005 versus corresponding value for WT mice.

showed comparable levels of mMCP-1 in their sera at day 14 (Fig. 6 B), suggesting that this late mMCP-1 induction is independent of endogenous IL-18 and possibly is dependent on Th2 cells generated thereafter.

Remarkable prolongation of *S. venezuelensis* L3 expulsion without help from Th2 cytokines and endogenous IL-18

To determine the relative contribution of Th2 cells and endogenous IL-18 to parasite expulsion, we compared the capacity of STAT6^{-/-} mice receiving control antibody or anti-IL-18 and/or anti-IL-2 antibodies to expel *S. venezuelensis*. STAT6^{-/-} mice inoculated with *S. venezuelensis* L3 increased their serum IL-18 levels during infection (Fig. 7 A). Inoculated STAT6^{-/-} mice required 23 d to expel parasites (Fig. 7 B), perhaps because of their inability to produce mMCP-1 promptly (Fig. 7 C). However, they started to produce mMCP-1 at day 14 and sustained substantial levels of mMCP-1 up to day 21 after infection (Fig. 7 C). Anti-IL-18 antibody treatment only modestly prolonged the time required for parasite expulsion (Fig. 7 B) and reduced serum mMCP-1 level (Fig. 7 C). However, STAT6^{-/-} mice injected with anti-IL-2/anti-IL-18 exhibited more profound defects in their ability to expel *S. venezuelensis* and to produce mMCP-1 than did STAT6^{-/-} mice (Fig. 7, B and C), although neutralization of IL-2 alone did not affect parasite expulsion and serum levels of

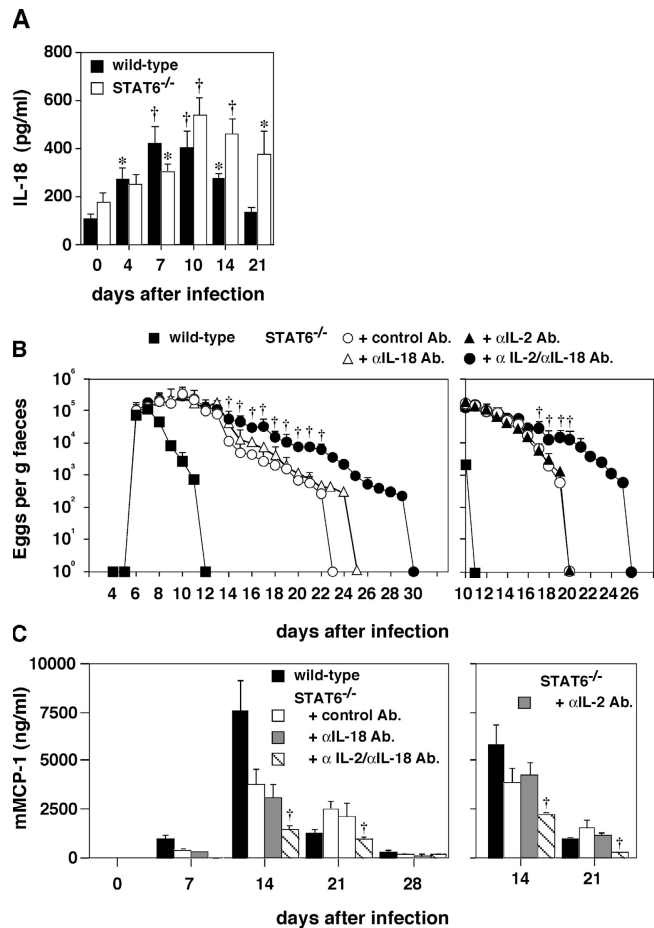


Figure 7. Role of Th2 cytokines and endogenous IL-18 against *S. venezuelensis* L3. (A) Serum levels of IL-18 from WT and STAT6^{-/-} mice inoculated with 5,000 *S. venezuelensis* L3. Results are geometric means ± SEM of five animals per group and are representative of three independent experiments. Ab, antibody; *, P < 0.01; †, P < 0.001 by Student's *t* test as compared with day 0. (B) Kinetics of the number of eggs per g feces and (C) serum levels of mMCP-1 from WT and STAT6^{-/-} mice inoculated with 5,000 *S. venezuelensis* L3. STAT6^{-/-} mice inoculated with *S. venezuelensis* L3 were injected intravenously with anti-IL-18 (800 μg/d) and/or anti-IL-2 (100 μg/d) or control antibody (rabbit IgG and rat IgG2b) two times per wk for 2 wk after infection. Results are geometric means ± SEM of five animals per group and are representative of three independent experiments. Ab, antibody; †, P < 0.005 versus corresponding value for STAT6^{-/-} mice injected with control antibody.

mMCP-1 (Fig. 7, B and C). These results taken together indicated involvement of two types of intestinal MMC activation, IL-18– plus IL-2–dependent and Th2 cell–dependent activation, for *S. venezuelensis* expulsion.

DISCUSSION

Here we show that pretreatment with IL-18 and IL-2 prepares mice to expel implanted adult worms promptly by induction of intestinal mastocytosis. We also show the relevant role of endogenous IL-18 for expulsion of *S. venezuelensis* L3

by using IL-18^{-/-} or IL-18R α ^{-/-} mice. Finally, we show the collaborative action of IL-18–dependently activated MMC and Th2 cell–dependently activated MMC for rapid parasite expulsion.

The role of intestinal MMC in worm expulsion has been studied extensively in various experimental host–parasite systems. In the case of infection with *S. venezuelensis* L3, host mice complete parasite expulsion within 2 wk, and the expulsion is tightly associated with level of intestinal mastocytosis (35, 36). In contrast, mast cell–deficient W/W^v mice infected with *S. venezuelensis* L3 show a significant delay in parasite expulsion (35). Furthermore, parasite expulsion is more severely impaired in W/W^v mice that are deficient for IL-3 gene expression. In these mice, MMC responses are almost completely absent, and *S. venezuelensis* continue to parasitize in the intestine for >50 d (35). In this study, we have demonstrated that depletion of CD4⁺ T cells in W/W^v mice further abrogated their capacity of to expel the parasite (Fig. 5 E). Thus, Th2 cells display their protective role against parasite even in the absence of activated MMC.

It is well established that mMCP-1, selectively expressed by intestinal MMC, participates in the effector–phase response to expulsion of intestinal nematodes (2, 26–29). Miller et al. reported that mMCP-1 is not detectable in the culture of BM-derived mast cells stimulated with IL-3 and IL-9 (40). However, mast cells begin to produce mMCP-1 when additionally stimulated with SCF and TGF- β . Here, we have demonstrated that daily injection of a mixture of IL-18 and IL-2 induces intestinal mastocytosis (Fig. 1 A) and an increase in the serum level of mMCP-1 (Fig. 2 A). This serum level of mMCP-1 is somewhat lower than that seen in WT mice infected with *S. venezuelensis* L3 for 14 d (Fig. 2 A). We have also demonstrated that mast cell–deficient W/W^v mice failed to produce mMCP-1 in response to the treatment with IL-18 and IL-2 or to the inoculation with *S. venezuelensis* L3 (Fig. 5 B). Based on these observations, we could conclude that MMC is the only producer of mMCP-1 in these circumstances.

Treatment of normal mice with IL-18 and IL-2 induced IL-3, IL-4, IL-9, and IL-13 (Fig. 1 C). Like WT mice treated with IL-18 plus IL-2, STAT6^{-/-} mice displayed intestinal mastocytosis and increased serum levels of mMCP-1 after IL-18 plus IL-2 treatment (Fig. 3, A and B). Finkelman et al. have previously reported that IL-4 treatment significantly increased the number of MMC in both WT and STAT6^{-/-} mice and that a much larger increase was observed in the latter, indicating that signaling through STAT6 seems to suppress IL-4 induction of MMC (33, 34). However, the IL-18–plus IL-2–induced mMCP-1 response in STAT6^{-/-} mice was inhibited only partly by blocking IL-4 (Fig. 3 C), suggesting contributions of other cytokines to mMCP-1 induction. Because CD4⁺ T cells stimulated by IL-18 plus IL-2 produce IL-3 and IL-9 as well as IL-4 and IL-13, we examined whether daily treatment with IL-3 (0.013 μ g/d) and IL-9 (0.5 μ g/d) for 2 wk could replace IL-18 and IL-2–treatment. We found

this treatment only partly replaced the effect of IL-2 plus IL-18 treatment on serum mMCP-1 level. However, a much higher dose of IL-3 (0.1 μ g/d), even without IL-9, replaced the effect of IL-2 and IL-18 (Fig. 3 E). Thus, we considered the possibility that a set of Th2 cytokines, including IL-3, IL-4, and IL-9, have an orchestrated action on mMCP-1 induction, and that neutralization of single factor could not effectively inhibit this orchestration.

In this report, we first showed that induction of intestinal mastocytosis by treatment with IL-18 plus IL-2 is sufficient to expel implanted adult worms. However, as we reported in the subsequent section, host defense against *S. venezuelensis* L3 infection is more complicated. We examined the contribution of endogenous IL-18 to parasite expulsion by using IL-18^{-/-} or IL-18R α ^{-/-} mice. WT mice infected with *S. venezuelensis* L3 showed a significant increase in serum levels of IL-18 (days 4–14) and mMCP-1 (days 7–21) and completed worm expulsion within 12 d (Fig. 6 A). In contrast, IL-18^{-/-} or IL-18R α ^{-/-} mice infected with *S. venezuelensis* L3 exhibited significantly reduced serum levels of mMCP-1 at days 4, 7, and 10 after infection, and worm expulsion was significantly delayed as compared with WT mice (Fig. 6 A). A reason for this delay might be that, like WT mice, both IL-18^{-/-} and IL-18R α ^{-/-} mice inoculated with *S. venezuelensis* L3 generated a Th2 response (unpublished data; reference 22). Thus, we assumed the possible contribution of Th2 cells to this late-phase worm expulsion.

IL-18 also is definitively involved in the host defense of STAT6^{-/-} mice. Because of their defective Th2 cell development, they required a much longer period to expel parasites. Anti-IL-2 or anti-IL-18 treatment alone showed little effect on parasite expulsion, whereas anti-IL-18 plus anti-IL-2 treatment markedly impaired the parasite expulsion and significantly reduced mMCP-1 production ($P < 0.005$; Fig. 7, B and C). Nevertheless, STAT6^{-/-} mice treated with anti-IL-2/anti-IL-18 still displayed mMCP-1 response after parasite infection. These results suggest participation of other factors in induction of mMCP-1. Recent reports have clearly demonstrated that nematode infection induces Th2 cytokines from basophils in the lung, liver, and spleen (41, 42), suggesting their contribution to mMCP-1 induction in STAT6^{-/-} mice.

As noted previously, neutralization of IL-18 and IL-2 showed profound effects on parasite expulsion, whereas neutralization of IL-2 or IL-18 alone showed very modest effects. At present, we have no explanation that accounts for this discrepancy. We considered the possibility that antibody neutralization of IL-18 might be incomplete, and residual IL-18 in combination with endogenous IL-2 might induce mMCP-1. Alternatively, an unidentified factor might partly replace the effect of IL-2 or IL-18. Thus, regulation of the mMCP-1 response is complicated, and we need to study further how STAT6^{-/-} mice expel infected parasites.

We suspected that IL-18 might be produced by various types of cells that are activated either directly or indirectly by

parasite infection. In fact, IL-18 is stored in bronchial and intestinal epithelium (43, 44), and it is quite possible that *S. venezuelensis* locally stimulates epithelial cells to produce IL-18.

Helmby and Grecis (45) have reported that daily injection of 0.2 μg of IL-18 into WT mice during infection with *Trichinella spiralis* decreased the mastocytosis as well as IL-13 secretion from mesenteric lymph node stimulated with *T. spiralis* antigen. Also, IL-18^{-/-} mice infected with *T. spiralis* developed a high level of mastocytosis in the intestine during infection, correlating with the rapid expulsion of the parasites. These results suggest that IL-18 negatively regulates intestinal mastocytosis and Th2 cytokine production. In sharp contrast, our results presented here reveal that pretreatment of normal mice with IL-18 and IL-2 induces significant recruitment and activation of intestinal MMCs, resulting in extremely rapid expulsion of adult *S. venezuelensis* worms. Our findings clearly indicate that IL-18 positively regulates intestinal mastocytosis in the presence of IL-2 and that treatment with IL-18 plus IL-2 has provided host mice with type 2 innate immunity that protects against gastrointestinal nematode infection.

There are several possibilities that account for these discrepancies. Studies have contrasted the role of IL-4 in STAT6^{-/-} mice infected with either *T. spiralis* or *Nippostrongylus brasiliensis*. *N. brasiliensis* infection of STAT6^{-/-} mice increase MMC and serum mMCP-1 levels, possibly through a primary Th2 response (33, 34). In contrast, MMC numbers and serum mMCP-1 responses were greatly decreased in STAT6^{-/-} mice infected with *T. spiralis*, and a reduced Th2 response resulted in lower IgG1 production (46). Therefore, type 2 cytokine responses are suppressed in STAT6^{-/-} mice infected with *T. spiralis*, even though these responses are normal in STAT6^{-/-} mice inoculated with *N. brasiliensis*. In other words, expulsion of *T. spiralis* is solely dependent on an STAT6-mediated signal that is required to stimulate the conditions that induce mast cells to degranulate optimally.

Helmby and Grecis reported that critical effects of IL-18 in *T. spiralis* infection are independent of IFN- γ and are mediated by the direct down-regulatory effect of IL-18 on IL-13 (45). It is important to point out that *N. brasiliensis* inoculation stimulates an almost pure type 2 cytokine response, whereas *T. spiralis* induces production of considerable IFN- γ in addition to IL-4. In this regard, adult *T. spiralis* live within various cells and induce considerably more IFN- γ production, probably through the induction of IL-12, than do *N. brasiliensis*, which live outside cells. Alternatively, *N. brasiliensis* may inhibit IL-12 production by DCs and macrophages. Therefore, it is possible that the work by Helmby and Grecis depicts a down-regulatory role of IL-18 on the Th2 response in the presence of IL-12 and its up-regulatory role on the Th1 response in the early phase of *T. spiralis* infections. In contrast to *T. spiralis* infection, *S. venezuelensis* infection resembles *N. brasiliensis* infection in its extracellular location in the host. It is possible that IL-2 is produced in the early phase of *S. venezuelensis* infection, which, along with endogenous IL-18, promotes MMC activation

and accumulation in the intestinal mucosa independently of STAT6-mediated signal.

Here, we present a new paradigm of IL-18 activity in the host mucosal immune response against gastrointestinal nematode infections. IL-18, together with IL-12, plays an important role in the elimination of intracellular infections (3, 18), and, with IL-2, it can eradicate gastrointestinal nematode infections by induction of functional intestinal MMCs. Thus, IL-18 plays an important role in the host defense against both intracellular and extracellular pathogens, and the cytokine milieu of IL-18 determines its final effect on infected parasites. At present, we have not yet identified the cell source of IL-18 in mice inoculated with *S. venezuelensis*. Our present results indicate that *S. venezuelensis* expulsion might be induced by two types of intestinal MMC activation, IL-18-dependent (innate type 2) MMC activation and Th2 cell-dependent (acquired type 2) MMC activation. We also suggest a strong contribution of IL-3 and IL-9 in inducing mMCP-1 production from MMCs.

MATERIALS AND METHODS

Mice and reagents. Specific pathogen-free male C57BL/6 mice and Wistar rats were obtained from Charles River Breeding Laboratories. Mast cell-deficient WBB6F1-W/W^v mice (37) and littermate control WBB6F1^{+/+} mice were purchased from SLC Japan. C57BL/6 RAG-2^{-/-} mice were obtained from Taconic. Generation of C57BL/6 STAT6^{-/-}, IL-18^{-/-}, and IL-18R α ^{-/-} mice has been detailed in our previous reports (47–49). All mice were bred under specific pathogen-free conditions at the animal facilities of Hyogo College of Medicine, Nishinomiya, Japan, and were used at 8 to 12 wk of age. All animal experiments were conducted according to the Guidelines for Animal Experiments at Hyogo College of Medicine. Recombinant human IL-2 was provided by Ajinomoto Co., Inc. Recombinant mouse IL-18 and IL-3/IL-9 were purchased from MBL International Corporation and from Genzyme. Anti-mouse IL-9 mAb (clone, D8402E8), biotinylated anti-mouse IL-9 mAb (clone, D9302C12), and anti-mouse IL-2 mAb (clone, S4B6, rat IgG2a) were purchased from BD Biosciences. Rabbit neutralizing anti-mouse-IL-18 IgG and control IgG antibodies were partially purified using Protein-A sepharose column in our laboratory. This anti-IL-18 antibody could completely neutralize 50 ng/ml of IL-18 at the concentration of 10 $\mu\text{g}/\text{ml}$ in vitro. The administration of 200 μg of anti-IL-18 antibody just before LPS challenge completely inhibited LPS-induced liver injury in mice (11). Rat antibodies against mouse IL-4 (11b11) and IL-4R α chain (M1) were purified in our laboratory. Intraperitoneal administration of a mixture of 10 mg of anti-IL-4 and 10 mg of IL-4R α antibodies 1 d before anti-IgD challenge completely inhibited anti-IgD-induced IgE production in vivo (50).

Parasites. *S. venezuelensis* has been maintained in serial passage in male Wistar rats in our laboratory. *S. venezuelensis* L3 were obtained from fecal culture by a filter paper method (36). Adult worms for intraduodenal implantation were prepared as follows. Wistar rats were inoculated with 30,000 infective larvae, and the upper half of the small intestines were removed 7 to 10 d after infection. The intestine was then cut open longitudinally and washed with PBS followed by incubation at 37°C for 80 min. Worms that emerged from the intestine were washed with sterile PBS and adjusted to an appropriate density. Adult worms suspended in 500 μl of PBS were injected into the duodenum of recipient mice (1,500 worms/mouse) under anesthesia using a 1-ml syringe with a 22-gauge needle. Animals were inoculated s.c. with 5,000 *S. venezuelensis* L3 to initiate a complete infection. The degree of infection of individual mice was monitored by counting the number of eggs excreted daily (eggs/g feces).

In vivo treatment of mice. C57BL/6 normal, C57BL/6 STAT6^{-/-}, RAG-2^{-/-}, WBB6F1^{+/+}, or WBB6F1-W/W^y mice were injected i.p. daily with PBS alone or with various combinations of IL-18 (0.5–2 µg/d) and/or IL-2 (2 × 10³–1 × 10⁴ U/d) for 13 d. To deplete CD4⁺ T cells, C57BL/6 mice were injected i.p. with monoclonal antibody to CD4 (clone, GK1.5; 0.5 mg/d; ATCC: TIB-207) or control antibody (rat IgG2b; 0.5 mg/d; BD Biosciences) 7 and 4 d before and 0, 3, and 7 d after IL-2 and/or IL-18 injection. For some experiments, C57BL/6 mice were injected daily with IL-3 (0.013–0.1 µg/d) and/or IL-9 (0.5 µg/d) for 13 d. These mice were killed on day 14 after cytokine treatments, and intestines, lungs, spleens, kidneys, and livers were removed for histological examination.

Intra duodenal adult worm implantation. To measure the invasion of adult *S. venezuelensis* into mouse intestinal mucosa, adult worms were implanted in the duodenum of recipient mice that had been treated with PBS or IL-2 and/or IL-18 for 13 d, and the mouse intestines were removed 16 h after implantation as described previously (36). Briefly, the intestine was cut open longitudinally and washed lightly to remove fecal matter and worms that were still in the lumen. Then the intestines were incubated at 37°C in PBS for 3 h. The number of worms that emerged from the intestines was counted. As we have reported previously, mucosal invasion of adult *S. venezuelensis* in naive host mice was completed within 4 h, and these implanted worms were rejected beyond 7 d after implantation in a mast cell-dependent mechanism (36). Based on these results, we recovered invading worms at 16 h after implantation.

ELISA. Mouse IL-9 was assayed by a specific sandwich ELISA with reference standard curves using known amounts of IL-9. To detect IL-9, we used anti-IL-9 mAb (D8402E8) and biotinylated anti-mouse IL-9 mAb (D9302C12; reference 22). The detection limit of this ELISA is 20 pg/ml. ELISA kits for IL-3, IL-4, and IL-13 (R&D Systems), and IL-18 (MBL International Corporation) were used. Levels of mMCP-1 in serum were measured with an mMCP-1 ELISA kit purchased from Moredun Scientific, Ltd.

Histological examination. For histological examination of MMC, tissue samples (8–10 cm) were removed from the pyloric ring. The intestine was opened longitudinally, flattened on a filter paper, and fixed in Carnoy's fluid and stained with Alcian blue, pH 0.3, and Safranin-O, as described previously (51). The number of mast cells in the epithelium and lamina propria mucosa was counted in 10 villous crypt units (VCU) according to the method described by Miller & Jarrett (52). Immunohistochemical staining for mMCP-1 on 6-µm-thick, paraffin wax-embedded, 10% formalin-fixed sections was performed as follows. The dewaxed sections were treated with 0.3% H₂O₂ in methanol for 30 min, then incubated with 10% normal rabbit serum in PBS for 1 h. The slides were incubated by sheep anti-mMCP-1 (Moredun Scientific, Ltd.) or normal sheep serum (Sigma-Aldrich) for 1 h, followed by incubation with peroxidase-conjugated rabbit anti-sheep IgG (Betty Laboratories, Inc.), and DAB reaction was performed.

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