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Pretreatment with a heat-killed probiotic modulates monocyte chemoattractant protein-1 and reduces the pathogenicity of influenza and enterovirus 71 infections

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It has been proposed that inactivated probiotics may modulate the host immune system and contribute to mitigation of viral infections. This study demonstrated that administration of heat-killed *Enterococcus faecalis*, a widely used probiotic, can protect host animals against viral infections. The influenza-mediated morbidity and lung inflammation in *E. faecalis*-treated mice decreased significantly compared with those of the control mice. Furthermore, we found that the protection is associated with production of monocyte chemoattractant protein-1 (MCP-1). The intratracheal injection of a recombinant mouse MCP-1 protein abrogated the antiviral effects elicited by pretreatment with *E. faecalis*. CC chemokine receptor 2 (CCR2) is a receptor for MCP-1, and the intraperitoneal administration of a CCR2 antagonist effectively inhibited viral pathogenicity. The reduced pathogenicity was also observed in CCR2-deficient mice. Finally, *E. faecalis* significantly attenuated neuropathogenicity induced by another RNA virus, enterovirus 71. This study demonstrates that killed probiotics can reduce viral disease severity and identify that the MCP-1 pathway might act as a key mediator in the improved antiviral immune response. Our findings suggest that MCP-1 and its related signaling pathway can serve as critical therapeutic targets for development of new antiviral strategies.

INTRODUCTION

Influenza A virus causes a highly contagious febrile respiratory illness characterized by seasonal (winter) epidemics and occasional pandemics.¹ The virions of influenza A virus, a member of the Orthomyxoviridae family, are enveloped particles containing eight negative-sense, single-stranded RNA segments comprising the viral genome. New options for preventing and controlling diseases caused by influenza A virus are urgently required.

Enterococcus faecalis is a Gram-positive, facultative anaerobic bacterium that belongs to the Lactobacillales clade. In recent years, *E. faecalis* has been widely used in diverse products (including fermented and non-fermented milks, infant formulas, and pharmaceutical preparations). Notably, *E. faecalis*

is part of the human gastrointestinal tract microbiota and can modulate immune function by acting as a probiotic.^{2,3} Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” by the Food and Agriculture Organization of the United Nations and the World Health Organization;⁴ however, mounting evidence indicates that not only live but also lysed lactic acid bacteria can exert immunomodulatory effects.⁵ Studies have demonstrated that heat-killed probiotics are safer than live probiotics for purposes such as eliminating antibiotic-resistant genes, preventing production of recombinant strains, controlling the microbial load during probiotic supplementation, and application in children and immunosuppressed patients.^{6,7} Furthermore, considerable clinical evidence has

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indicated that dead probiotic bacteria can improve health; for example, they can enhance immunity in elderly people;⁸ they have benefits as a complementary therapy for adult atopic dermatitis patients receiving the conventional treatment;⁹ they can effectively improve the overall quality of life for patients with allergic rhinitis;¹⁰ and they reduce the incidence of upper respiratory tract infection in healthy people with high levels of psychological stress.¹¹ Specifically, it has been previously reported that oral administration of lysed *E. faecalis* to mice can reduce both the allergen-induced peritoneal accumulation of eosinophils¹² and the serum ratio of allergen-specific IgE to IgG2a antibodies.¹³ Another study demonstrated that lysed *E. faecalis* can restore an intestinal ecosystem disrupted by antibiotics, preventing the subsequent development of atopy.¹⁴ Although pretreatment with viable or inactivated probiotics has been shown to have protective effects against influenza virus infection,^{15,16} the potential mechanisms underlying this anti-influenza protection are still poorly understood. In addition, the question as to whether heat-killed probiotics can improve anti-influenza immunity remains unanswered.

In addition, infections by enterovirus 71 (EV71), a member of the Picornaviridae family, have recently emerged as an important public health concern in the Asia-Pacific region, where they can lead to severe neurological disorders (e.g., brainstem encephalitis, meningitis, and poliomyelitis-like paralysis) and death.^{17,18} In this study, we tested whether heat-killed probiotics can modulate the effect on the host response to viral infections using either influenza or EV71 viruses.

Here, we investigated whether heat-killed *E. faecalis* can improve the immune response of mice against influenza A virus or EV71 infection. Our results indicated that monocyte chemoattractant protein-1 (MCP-1) and its associated immunomodulatory pathway are critical for *E. faecalis*-mediated antiviral effects.

RESULTS

Oral administration of heat-killed *Enterococcus faecalis* before infection protects mice against influenza A virus-related symptoms

We first investigated whether oral administration of heat-killed *E. faecalis* for 12 days before experimental infection may protect C57BL/6 mice against influenza A virus-related clinical symptoms (i.e., mortality and weight loss). After intranasal infection with the WSN influenza A virus strain, we compared the clinical symptoms of mice that received heat-killed *E. faecalis* or the vehicle (**Figure 1a**). The survival rates in the *E. faecalis*- and vehicle-treated groups were ~50 and 75%, respectively (**Figure 1b**). After 8 days of infection, mice treated with *E. faecalis* lost $8.4 \pm 6.7\%$ of their initial body weight as compared with a $23.4 \pm 5.2\%$ loss observed in the vehicle group ($P < 0.05$; **Figure 1c**). *E. faecalis* *per se* had no effect on death or weight loss in uninfected mice. After killing the mice, lung viral titers were measured at 4, 7, and 10 days post infection. In *E. faecalis*-treated mice, high viral titers on day 4 were followed by a significant decrease on day 7 (**Figure 1d**). Moreover, lung

viral titers were significantly lower in *E. faecalis*-treated mice than in the vehicle group. The inflammatory lung reaction was assessed by measuring leukocyte infiltration in the bronchoalveolar lavage (BAL) fluid. Infection with influenza A virus induced transient immune cell infiltration, which was characterized by an earlier onset and a longer duration in the vehicle group than in *E. faecalis*-treated mice (**Figure 1e**). A persistent infiltration of neutrophils, lymphocytes, and monocytes was observed in vehicle-treated mice for 7–10 days post infection, whereas the *E. faecalis*-fed group showed a negligible inflammatory reaction at day 7 (**Figure 1f**). Basophils and eosinophils were rarely observed ($<1\%$ of all cells) in inflammatory infiltrates, and no significant intergroup differences were noted. Taken together, these findings indicate that heat-killed *E. faecalis* may protect mice when administered orally before experimental influenza A virus infection. In addition, we tested whether live *E. faecalis* also protects mice against influenza A virus infection. The results (**Figure 2**) indicated that the antiviral effect of live *E. faecalis* was similar to that observed for heat-killed *E. faecalis*. Taken together, our data demonstrate that not only live *E. faecalis* but also heat-killed *E. faecalis* can protect mice when administered orally before experimental influenza A virus infection.

Oral administration of heat-killed *Enterococcus faecalis* after viral infection does not protect mice against influenza A virus-related clinical symptoms. We next assessed whether oral administration of heat-killed *E. faecalis* after experimental influenza A virus infection can prevent or delay the onset of clinical symptoms. One hour after infection, mice were orally intubated once-daily with phosphate-buffered saline (PBS) (vehicle) or *E. faecalis* (**Figure 3a**). No significant intergroup differences in terms of survival rates, weight loss, virus titers in the lung, or leukocyte infiltration in BAL were identified (**Figure 3**). Consequently, the antiviral effects of heat-killed *E. faecalis* were observed only when it was orally administered before influenza A virus infection.

Cytokines involved in heat-killed *Enterococcus faecalis*-induced immunomodulation after influenza A virus infection *in vivo* and *in vitro*. As inflammatory cytokines have a key role in the immune response to influenza virus infections,¹⁹ we further evaluated how *E. faecalis* modulates the virus-induced cytokine response both *in vivo* and *in vitro*. We initially evaluated whether inoculation of heat-killed *E. faecalis* could alter BAL cytokine and chemokine concentrations in the absence of viral infections. Thus, mice were intubated with *E. faecalis* once-daily for 12 consecutive days. BAL levels of IL-6, TNF- α , IFN- γ , IL-10, IL-1 β , IL-17, MCP-1, and TGF- β were measured by immunoassays. However, all of these molecules were undetectable in both *E. faecalis*-fed and control mice (data not show). These findings suggest that *E. faecalis* *per se* does not alter BAL cytokine and chemokine concentrations.

To assess whether heat-killed *E. faecalis* can modulate virus-induced cytokine production *in vivo*, BAL cytokine and chemokine concentrations were measured on days 4, 7, and 10 post infection (**Figure 4a**). In general, influenza A virus

Figure 1 Heat-killed *Enterococcus faecalis* protects against influenza A virus infection when administered orally before virus infection. **(a)** Schematic representation of the experimental setup. After oral intubation of C57BL/6 male mice, phosphate-buffered saline (PBS) (vehicle) or heat-killed *Enterococcus faecalis* was given once-daily for 12 consecutive days before experimental infection with influenza A virus (1×10^3 plaque-forming units (PFU)). **(b)** The percent survival was calculated on a daily basis. Survival curves were compared using the Gehan–Breslow–Wilcoxon test. **(c)** Body weight was measured daily after the experimental infection, and all data were normalized to the initial weight of each mouse. Error bars represent the standard error of mean (s.e.m.), total number ($n \geq 5$ mice per group, * $P < 0.05$ (two-way repeated-measures ANOVA followed by the Tukey's *post hoc* test). **(d)** Lung viral titers were determined at the indicated time points post-infection using the plaque assay (five mice per group). Error bars represent the s.e.m., * $P < 0.05$ (two-way ANOVA followed by the Bonferroni's *post hoc* test). n.d., not detected. **(e, f)** Total cell and differential cell counts in bronchoalveolar lavage (BAL) specimens were obtained using standard blood count methods. * $P < 0.05$, ** $P < 0.01$ ($n = 3$ –5 mice per group, two-tailed Student's *t*-test). EF, *Enterococcus faecalis*; d.p.i., days post-infection; WSN, an influenza A virus strain (A/WSN/33).

infection increased levels of proinflammatory molecules in BAL, regardless of pretreatment with heat-killed *E. faecalis*. However, BAL concentrations of both IL-6 and MCP-1 were significantly lower in the *E. faecalis*-treated group at day 10 post infection (**Figure 4b**).

We also evaluated whether inoculation of heat-killed *E. faecalis* can enhance macrophage antiviral activity *in vitro*. After inoculation of heat-killed *E. faecalis*, peritoneal macrophages were isolated, cultured, and infected with different doses (multiplicity of infection of 5 and 10) of influenza A virus (**Figure 4c**). We quantified the concentrations

of IL-6, TNF- α , IFN- γ , IL-10, IL-1 β , IL-17, MCP-1, and TGF- β in cell-culture supernatants at 0, 3, 6, 16, and 24 h post infection. Cell-culture supernatants of macrophages isolated from mice pretreated with heat-killed *E. faecalis* were characterized by higher levels of IL-17 and TNF- α and lower concentrations of MCP-1 and IL-1 β (**Figure 4d**). However, IL-6, IFN- γ , IL-10, and TGF- β were undetectable in cell-culture supernatants (data not shown).

MCP-1 is a potent chemotactic factor capable of inducing monocyte migration and their differentiation into macrophages.^{20,21} Although MCP-1 is involved in the immune

Figure 2 Live *Enterococcus faecalis* protects mice against influenza A virus infection when administered orally before virus infection. **(a)** Schematic representation of the experimental setup. After oral intubation of C57BL/6 male mice, phosphate-buffered saline (PBS) (vehicle) or live *Enterococcus faecalis* was administered once-daily for 12 consecutive days before experimental infection with influenza A virus (1×10^3 plaque-forming units (PFU)). **(b)** Percent survival was calculated on a daily basis. Survival curves were compared using the Gehan–Breslow–Wilcoxon test. **(c)** Body weight was measured daily after the experimental infection, and all data were normalized to the initial weight of each mouse. Error bars represent the standard error of the mean (s.e.m.), total number (n) = 5 mice per group, $*P < 0.05$ (one-way repeated-measures ANOVA followed by the Bonferroni's *post hoc* test). **(d)** Lung viral titers were determined at the indicated time points post-infection by using the plaque assay (5 mice per group). Error bars represent the s.e.m., $*P < 0.05$ (two-way ANOVA followed by the Bonferroni's *post hoc* test). **(e, f)** Total cell and differential cell counts in bronchoalveolar lavage (BAL) specimens were obtained using standard blood count methods. $*P < 0.05$, $**P < 0.01$ ($n = 4$ –5 mice per group, two-tailed Student's *t*-test). n.d., not detected.

response against influenza virus infection, its excess production has been linked to progressive lung inflammatory disorders.^{22–24} Moreover, increased IL-1 β levels have been shown to contribute to immune pathology following influenza A virus infection.²⁵ Consequently, the lower levels of MCP-1 and IL-1 β observed in the *E. faecalis*-treated mice group may be associated with a blunted inflammatory response in the lung following viral infections.

We next investigated whether heat-killed *E. faecalis* can influence MCP-1 gene expression in macrophages in the absence of virus infections. To this aim, mice were intubated with heat-killed *E. faecalis* once-daily for 12 consecutive days. However, the expression levels of MCP-1 mRNA in peritoneal macrophages did not differ significantly between *E. faecalis*-fed and control animals (**Supplementary Figure S1** online). These results indicate that the administration of heat-killed *E. faecalis*

Figure 3 Oral administration of heat-killed *Enterococcus faecalis* after experimental infection does not protect mice against influenza A virus-related clinical symptoms. **(a)** Schematic representation of the experimental setup. One hour after infection with influenza A virus (1×10^3 plaque-forming units (PFU)), mice were orally intubated once-daily with phosphate-buffered saline (PBS) (vehicle) or *E. faecalis* for 7 days after. Experiments were conducted in ≥ 5 mice per group. **(b)** The percent survival was calculated on a daily basis. **(c)** Body weight was measured daily after infection, and data were normalized to the initial weight of each mouse. Error bars represent the standard error of mean (s.e.m.). **(d)** Lung or tracheal viral titers were determined on day 7 post infection using the plaque assay. Error bars represent the s.e.m. **(e)** Total leukocyte count in bronchoalveolar lavage (BAL) was obtained on day 7 post infection. *** $P < 0.001$ (two-tailed Student's *t*-test).

per se does not exert a significant influence on MCP-1 expression in the absence of viral infections.

Given the well-known role of MCP-1 as a chemoattractant, we expectedly observed a lower number of monocytes/lymphocytes in BAL of the *E. faecalis*-fed group (**Figure 1f**). These results highlight the role of MCP-1 in *E. faecalis*-elicited immunomodulation following influenza A virus infection.

The impact of MCP-1/CCR2 pathway on the heat-killed *Enterococcus faecalis* evoked antiviral ability in influenza virus-infected mice. We then sought to further dissect the potential role of MCP-1 in the prevention of influenza virus-mediated lung injury elicited by heat-killed *E. faecalis*. We therefore examined whether intratracheal injection of the rmMCP-1 protein could reverse the antiviral effects of *E. faecalis* (**Figure 5a**). *E. faecalis* reduced mortality in mice following influenza A virus infection, but this protective effect was abrogated when rmMCP-1 was administered concomitantly (**Figure 5b**). Similar findings were observed when weight loss was considered (**Figure 5c**). *E. faecalis* reduced viral titer and leukocytes infiltration in their lung was also abrogated when rmMCP-1 was administered concomitantly (**Figure 5d,e**).

Because MCP-1 acts through the CC chemokine receptor 2 (CCR2), a CCR2 antagonist (RS102895) that binds with high

affinity to the β subunit of the CCR2 receptor effectively blocks CCR2 signaling.²⁶ We therefore examined whether blocking CCR2 signaling could affect clinical symptoms after experimental infection with influenza A virus (**Figure 5f**). The intraperitoneal injection of RS102895 abrogated influenza-associated mortality, with a survival rate of 100% being observed in treated mice (**Figure 5g**). Similar findings were observed when weight loss was considered (**Figure 5h**). In addition, mice treated with RS102895 had significantly reduced viral titer and leukocytes infiltration in their lungs compared with the vehicle group (**Figure 5i,j**).

Taken together, we observed that (i) rmMCP-1 abrogated heat-killed *E. faecalis*-induced suppression of influenza-mediated lung injury and (ii) the use of RS10289 prevented viral pathogenicity. These results suggest that MCP1 administration can reverse the antiviral effect of *E. faecalis* and that excess MCP1 may be relevant to influenza pathogenesis.

CCR2-knockout mice are characterized by decreased mortality and lower weight loss after experimental influenza A virus infection. To further confirm the role of MCP-1 in the antiviral response, we compared the effects of experimental influenza A virus infection in CCR2-deficient and wild-type mice. CCR2-deficient

We investigated whether oral administration of equivalent Toll-like receptor (TLR) ligands could elicit antiviral effects similar to that observed with heat-killed bacteria. *E. faecalis* is

Figure 5 Impact of the monocyte chemoattractant protein-1 (MCP-1)/CC chemokine receptor 2 (CCR2) pathway on the antiviral activity of heat-killed *Enterococcus faecalis* in influenza virus-infected mice. (a) Schematic representation of the experimental setup. C57BL/6 male mice were orally intubated once-daily with phosphate-buffered saline (PBS) (vehicle) or *Enterococcus faecalis* for a total of 12 days before influenza A virus infection. Infected mice received intratracheal injections of rmMCP-1 protein (10 μ g kg⁻¹) or vehicle once-daily for 5 consecutive days. (b, g) Percent survival was calculated on a daily basis. Survival curves were compared using the Gehan–Breslow–Wilcoxon test (5–7 mice per group). (c, h) Body weight was measured daily after infection and data were normalized to the initial weight of each mouse (5–7 mice per group; two-way repeated-measures ANOVA followed by the Tukey's *post hoc* test). (d, i) Lung viral titers were measured at 7 days post infection by using the plaque assay (4–5 mice per group, one-way ANOVA followed by the Tukey's *post hoc* test). (e, j) Total cell counts in bronchoalveolar lavage (BAL) specimens were obtained using standard blood count methods (4–5 mice per group, one-way ANOVA followed by the Tukey's *post hoc* test). (f) Schematic representation of the experimental setup. The CCR2 antagonist (RS102895, 5 mg kg⁻¹ day⁻¹) or vehicle was injected into the peritoneal cavity after experimental infection, once-daily for 5 consecutive days. EF, *Enterococcus faecalis*; V, vehicle; rmMCP-1, recombinant mouse MCP-1; antagonist, CCR2 antagonist, RS102895. Error bars represent the standard error of the mean. * $P < 0.05$, EF vs. V; ## $P < 0.05$, EF + rmMCP-1 vs. EF alone; # $P < 0.05$, antagonist vs. V; and ** $P < 0.05$, V vs. V + rmMCP-1.

Figure 6 CC chemokine receptor 2 (CCR2)-knockout mice exhibited decreased mortality, less body weight loss, reduced virus titer, and reduced leukocyte infiltration in the lung. **(a)** Percent survival was calculated on a daily basis. Survival curves were compared using the Gehan–Breslow–Wilcoxon test. **(b)** Body weight was measured daily after infection, and data were normalized to the initial weight of each mouse. * $P < 0.05$ (two-way repeated-measures ANOVA followed by the Tukey's *post hoc* test). **(c)** Lung viral titers were determined at the indicated time points post-infection by using the plaque assay. * $P < 0.05$ (two-way ANOVA followed by the Tukey's *post hoc* test). **(d, e)** Total cell and differential cell counts in bronchoalveolar lavage (BAL) specimens were obtained using standard blood count methods. ** $P < 0.01$ (two-tailed Student's *t*-test). Error bars represent the standard error of the mean (s.e.m.), total number ($n = 5$ – 6 mice per group, n.d., not detected).

a Gram-positive bacterium, and lipoteichoic acid (LTA) is a major constituent of its cell wall. Consequently, LTA and bacterial DNA (isolated from *E. faecalis*) were used as ligands of TLRs 2 and 9, respectively. We calculated that 8.5×10^{10} colony-forming unit (CFU) $\text{kg}^{-1} \text{day}^{-1}$ of heat-killed bacteria corresponded to ~ 170 and $165 \mu\text{g kg}^{-1} \text{day}^{-1}$ of LTA and bacterial DNA, respectively. **Supplementary Figure S2** shows that the LTA-treated group exhibited less weight loss, lower viral titers, and decreased levels of leukocyte infiltration compared with untreated animals. However, these beneficial effects were not observed in the DNA-treated group. Because LTA can partially protect mice when administered orally before experimental influenza A virus infection, our data suggest that orally administered TLR 2 ligands may be able to exert antiviral activity in mice.

Oral administration of heat-killed *Enterococcus faecalis* before experimental infection protects against EV71 infection. Finally, we assessed whether administration of heat-killed *E. faecalis* could prevent the neurological complications induced in neonatal mice by experimental EV71 infection. Ten-day-old mice are highly susceptible to EV71 infection and develop clinically relevant symptoms (e.g., hunchback and limb weakness), which ultimately progress to rear limb paralysis. Five-day-old ICR mice were therefore orally intubated once per day with PBS (vehicle) or heat-killed *E. faecalis* for 5 consecutive days and then infected with EV71 at day 10 (**Figure 7a**). The clinical scoring method for assessing the severity of enterovirus infections consists of five clinical parameters that assess motor function and movement behavior.²⁷ Clinical scores were assessed on a daily basis after the experimental infection.

Paresis was initially mild and characterized by decreased limb movements. Total paralysis developed in the subsequent 6–7 days (**Figure 7b**). Oral administration of heat-killed *E. faecalis* significantly protected mice against EV71-induced neurotoxicity, with treated mice showing only ruffled hair and limb weakness. The growth retardation induced by EV71 infection occurred earlier and showed greater severity in vehicle-treated mice as compared with animals pretreated with heat-killed *E. faecalis* (**Figure 7c**). These results suggest that *E. faecalis* is capable of eliciting an effective antiviral immune response within a short time of administration.

Because EV71 is known to cause neuroinflammation,²⁸ we sought to identify viral particles in the central nervous system of experimentally-infected mice. Infectious particles were evident in all of the brain areas being examined at all time points. Interestingly, they were predominantly located in the spinal cord (**Figure 7d**). Administration of heat-killed *E. faecalis* did not completely eliminate the presence of infectious viral particles, which were nonetheless less frequent (as compared with vehicle-treated animals) in the spinal cord, brainstem, cerebellum, motor cortex, and hippocampus. Overall, these data suggest that orally administered heat-killed *E. faecalis* can positively regulate antiviral immunity against EV71 infection.

We next investigated whether administration of *E. faecalis* after EV71 infection could ameliorate clinical score in neonatal mice. One hour after experimental infection, 10-day-old ICR mice were fed either PBS (vehicle) or heat-killed *E. faecalis* (*E. faecalis*-fed group) once-daily (**Supplementary Figure S3A**). The two groups did not differ significantly in terms of EV71 virus-induced paralysis (**Supplementary**

Figure 7 Oral administration of heat-killed *Enterococcus faecalis* protects against enterovirus 71 (EV71) infection. (a) Schematic representation of the experimental setup. Five-day-old ICR mice were orally intubated once-daily with phosphate-buffered saline (PBS) (vehicle) or *Enterococcus faecalis* for 5 consecutive days before EV71 infection (≥ 5 mice per group). (b) Clinical scores were determined daily after infection. Error bars represent the standard error of mean (s.e.m.) and $*P < 0.05$ (two-way repeated-measures ANOVA followed by the Tukey's *post hoc* test). (c) Body weight was measured daily after EV71 infection, and the data were normalized to the initial body weight of each mouse. Error bars represent the s.e.m. $*P < 0.05$ (two-way repeated-measures ANOVA followed by the Tukey's *post hoc* test). (d) Viral titers in the brain and different tissues were determined in rhabdomyosarcoma (RD) cells at the indicated time points after infection using the plaque assay. Error bars represent the s.e.m. $*P < 0.05$, $**P < 0.01$ (two-way repeated-measures ANOVA followed by the Tukey's *post hoc* test).

Figure 2B) or growth retardation (Supplementary Figure S3C). Moreover, the number and distribution of infectious viral particles in the central nervous system did not show significant intergroup differences (Supplementary Figure S3D). Consequently, the antiviral effects of heat-killed *E. faecalis* were observed only when it was administered before EV71 infection.

MCP-1 is involved in the *E. faecalis*-mediated attenuation of EV71 virus infection. Proinflammatory cytokines have a pivotal role in mediating the neuropathological effects of EV71 infection,²⁹ including neuronal loss.^{30,31} Thus, we measured expression levels of IL-6, TNF- α , IFN- γ , IL-10, IL-1 β , IL-17, MCP-1, and TGF- β . Consistent with previous results, EV71 infection was associated with an increased expression of different cytokines in both the spinal cord and the serum (Figure 8a,b). Notably, MCP-1 expression was significantly downregulated by pretreatment with heat-killed *E. faecalis*. We therefore investigated the effect of intraperitoneally-injected rmMCP-1 in mice receiving heat-killed *E. faecalis* before experimental infection with EV71. The injection of rmMCP-1 in EV71-infected mice abrogated both the neuroprotective action of *E. faecalis* (Figure 8c) and its effect against growth inhibition (Figure 8d).

These data again prove that killed probiotics can reduce viral disease severity and identify the MCP-1 pathway as a key mediator in the improved antiviral immune response.

DISCUSSION

In this study, we demonstrate that not only live but also heat-killed *E. faecalis* can protect animals against RNA virus infections (specifically caused by influenza virus and EV71) and may modulate MCP-1 production. The Gram-positive probiotic bacterium *E. faecalis* contains LTA components in its cell wall and genomic DNA in its nuclei. These two components can be released into the gut after its oral administration, leading to the stimulation of TLRs. Specifically, TLR2 and TLR9 act as receptors for LTA and DNA, respectively. Our findings demonstrate that equivalent amounts of a TLR2 ligand (LTA) administered orally improve mice response to viral infections, whereas oral equivalents of TLR9 ligands did not. We thus speculate that heat-killed *E. faecalis* may exert its antiviral activity through the modulation of TLR2 signaling pathway.

The binding of pathogen-associated molecular pattern (PAMP) ligands to TLRs activates a signaling cascade involving the interaction of the cytoplasmic TIR (Toll/IL-1 receptor) domain that associates with a TIR domain-containing adaptor,

Figure 8 The impact of monocyte chemoattractant protein-1 (MCP-1)/CC chemokine receptor 2 (CCR2) pathway on the heat-killed *Enterococcus faecalis* evoked antiviral ability in enterovirus 71 (EV71)-infected mice. (a, b) Cytokine and chemokine concentrations in the spinal cord and serum were measured using enzyme-linked immunosorbent assays (ELISAs) (5 mice per group). * $P < 0.05$ (two-tailed Student's *t*-test). (c, d) Body weight and clinical scores were measured daily after EV71 infection. Error bars represent the standard error of mean (s.e.m.). Body weight data were normalized to the initial body weight of each mouse. $n = 5$ mice per group, * $P < 0.05$, *E. faecalis* vs. vehicle; and # $P < 0.05$, *E. faecalis* + recombinant mouse MCP-1 (rmMCP-1) vs. *E. faecalis* (two-way repeated-measures ANOVA followed by the Tukey's *post hoc* test).

MyD88 (myeloid differentiation primary response 88), which leads to the regulation of the transcription of proinflammatory mediators, particularly in innate immune cells.³² Recent studies have demonstrated that these proinflammatory mediators can elicit antiviral effects.^{33,34} First, LTA treatment can upregulate the expression of MyD88, iNOS, and IL-1 β and can reduce infectious laryngotracheitis virus plaques in embryonic eggs.³³ Second, the activation of TLR2 from LTA enhances cathelicidin expression in mast cells, thereby enhancing their capacity to fight the vaccinia virus.³⁴ In addition, a study demonstrated that MCP-1 expression is induced in murine macrophages by the activation of TLR2 from LTA purified by *E. faecalis*.³⁵ An interesting question is how administration of heat-killed *E. faecalis* can protect host animals against infection by two unrelated viruses. According to our data from this study, we speculate that the TLR ligands produced by components of bacteria may modulate TLR signaling, thereby controlling the expression level of proinflammatory cytokines, especially TLR2 and its downstream chemokine MCP-1.

MCP-1 is a widely expressed chemoattractant of monocytes and macrophages.³⁶ In the lung, MCP-1 elicits an early inflammatory response accompanied by an increased synthesis of cytokines and recruitment of neutrophils, which is followed by a late phase of inflammation characterized by enhanced monocyte trafficking and expansion of the alveolar macrophage pool.³⁷ A previous study has also shown that MCP-1 has a key role in orchestrating a protective immune response during influenza virus infection.²⁴ Conversely, MCP-1 and its hematopoietic cell receptor CCR2 are expressed at high levels in inflammatory lung disorders.²² Taken together, these findings

suggest that MCP-1 expression is tightly regulated during inflammation and viral infections.

Our results indicate that oral administration of heat-killed *E. faecalis* before viral infection suppressed influenza virus-related weight loss in mice, a beneficial effect that was abrogated by the intratracheal injection of rmMCP-1. Conversely, intraperitoneal administration of the CCR2 antagonist RS102895 inhibited influenza virus-related clinical symptoms. Our findings confirm and expand previous data, indicating that CCR2-deficient mice are less prone to influenza virus-induced morbidity and mortality,^{38,39} possibly because of a reduced monocyte trafficking in the lungs. In the central nervous system, MCP-1 is involved in neuroinflammation by acting as a chemoattractant for microglia.²¹ Importantly, our study also demonstrates a significant protective effect of *E. faecalis* against EV71-mediated growth retardation in mice. In particular, the intraperitoneal injection of rmMCP-1 abrogated the antiviral effects elicited by *E. faecalis* administration. These results indicate that orally administered heat-killed *E. faecalis* may mitigate EV71-induced clinical score through MCP-1 regulation. Taken together, our findings suggest that MCP-1 could serve as a critical therapeutic target for devising new antiviral strategies.

Immune-mediated pathology has a critical role in both influenza-related symptoms^{19,38} and EV71-mediated neurological disorders.²⁹ In our study, pretreatment with *E. faecalis* reduced influenza-associated weight loss, MCP-1 production, and immune cell infiltration in the lung. Moreover, *E. faecalis*-treated animals showed a blunted MCP-1 response and a reduced viral load after EV71 infection. Fine tuning of the

innate immune system by heat-killed *E. faecalis* may thus optimize the host antiviral defences.⁴⁰

IL-17 is a potent regulator of neutrophil responses and is involved in the pathogenesis of acute lung injury. Viral infections are frequently characterized by increased IL-17 levels, which can boost the proinflammatory outcome of antiviral responses.^{41,42} In addition, an increased TNF- α expression can limit the magnitude of the immune responses and mitigate viral-induced immunopathology.⁴³ Consequently, the increased production of IL-17 and TNF- α elicited by heat-killed *E. faecalis* suggests its potential usefulness for accelerating viral clearance.

Heat-killed *E. faecalis* is currently being marketed as a probiotic. A previous study demonstrated that *E. faecalis* strains isolated from newborn babies can regulate endogenous peroxisome proliferator-activated receptor gamma activity and IL-10 levels in colonic epithelial cells.⁴⁴ Moreover, *E. faecalis* may have a role in immunomodulation through the stimulation of nitric oxide production via activation of TLR-2 and TLR-1 or TLR-6 heterodimers.⁴⁵ Finally, *E. faecalis* can protect against IgE-mediated allergy, reduce eosinophil accumulation,^{12,13} and can prevent gut dysbiosis caused by antibiotic use.¹⁴

In the current report, we demonstrate that heat-killed *E. faecalis* can protect animals against experimental RNA virus infections (specifically caused by influenza virus and EV71) and modulates MCP-1 production. It is noteworthy that heat-killed and not live bacteria were used for this study. In addition, considerable clinical evidence has indicated that dead probiotic bacteria can improve health for many patients, including elderly people,⁸ adult atopic dermatitis patients,⁹ and such bacteria reduce the incidence of infection in people with high levels of psychological stress.¹¹ Previous research suggests that the immune responses elicited by probiotics may differ according to the growth phase (i.e., logarithmic vs. stationary) and may differ depending on the use of live vs. heat-killed bacteria.⁴⁶ Notably, the clinical use of viable probiotics is not without caveats, including the potential carriage of antibiotic-resistance genes and potential safety issues in at-risk populations (e.g., children, frail elderly subjects, and immunosuppressed patients).⁷ In this scenario, heat-killed probiotics might not only represent a safer alternative than live bacteria but can also be more convenient for preparation and administration (for example through a strict control of the microbial load).⁴⁷

In summary, the results from the current study indicate that pretreatment with heat-killed *E. faecalis* can modulate MCP-1 and protect mice against infections caused by two unrelated viruses (i.e., influenza virus and EV71). These results suggest that these heat-killed probiotics could boost immunity against viral infections in general. Further clinical studies on the potential antiviral activity of heat-killed *E. faecalis* in humans are warranted.

METHODS

Ethics statement. All animal experiments were conducted in accordance with the policies and procedures set forth by the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of

Health. All procedures were approved by the Chang Gung University review committee (IACUC approval number CGU11-004).

Mice. Male CCR2-deficient mice were purchased from Jackson Laboratory (Bar Harbor, ME). Male C57BL/6 mice and ICR mice were obtained from BioLasco Biotechnology Company (Taipei, Taiwan). Mice were maintained in environmentally controlled rooms (controlled room temperature: $25 \pm 1^\circ\text{C}$) kept in 12 h light-dark cycles.

Viral infection of mice. For the influenza infection model, CCR2-deficient and C57BL/6 mice aged 7 weeks old were used. The isoflurane-anesthetized mice were intranasally infected with a median lethal dose (LD_{50} , 1×10^3 plaque-forming units (PFU) per mouse) of an influenza A virus strain (A/WSN/33) in 50 microliter of PBS per mouse. After challenge, changes in body weight and mortality were carefully monitored. In compliance with ethical standards and regulations for animal experiments, the end point “mortality” was considered to be reached when a weight loss of $> 25\%$ from the initial value occurred. On days 4, 7, or 10 post challenge, mice were killed and specimens were collected for further analysis (to evaluate protection from infection). All mice were humanely killed through an overdose of isoflurane and carefully observed and palpated to confirm the absence of respiration and heartbeats. For the EV71 infection model, 10-day-old ICR mice were injected intraperitoneally with 5×10^5 PFU of a mouse-adapted EV71/MP4 strain derived from the parental strain EV71/Tainan/4643/98. After challenge, all mice were monitored daily over 7 days to measure clinical scores and investigate changes in body weight.

Virus and cell. The Madin-Darby canine kidney (MDCK) cells and RD (rhabdomyosarcoma) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) medium supplemented with 10% fetal bovine serum (FBS) (Gibco, NY). Cells were maintained at 37°C under conditions of 5% CO_2 . The influenza A/WSN/33(H1N1) virus was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and grown on the MDCK cells by using standard methods. The EV71/MP4, a mouse-adapted strain derived from the parental virus EV71/Tainan/4643/98 (GenBank accession number AF304458), was grown in RD cells.

Preparation and administration of *E. faecalis* in mice. *E. faecalis* KH2 (Cosmo Foods, Tokyo, Japan)—originally isolated from a fruit—was routinely cultured at 37°C for 16 h in brain heart infusion (BHI, Oxoid, Basingstoke, UK) broth and resuspended in sterile endotoxin-free water (HyClone, Logan, UT). Bacteria were serially diluted to measure both the strain-specific densities (optical density at 600 nm) and the yield CFUs (CFU ml^{-1}) by plate counting. The suspension of killed bacteria was heated to 80°C for 30 min. Oral intubation for intragastric administration of *E. faecalis* (either killed or live) or the vehicle was performed at a dosage of $17 \text{ mg kg}^{-1} \text{ day}^{-1}$ (equivalent to $8.5 \times 10^{10} \text{ CFU kg}^{-1} \text{ day}^{-1}$).

LTA, DNA, RS102895, and rmMCP-1 administration. Intraperitoneal injections of the CCR2 antagonist RS102895 (Sigma-Aldrich, St Louis, MO; $5 \text{ mg kg}^{-1} \text{ day}^{-1}$ in 0.005% dimethyl sulfoxide) or the vehicle were performed once-daily for 5 consecutive days after virus infection. Influenza virus- and EV71-infected mice received intratracheal and intraperitoneal injections of rmMCP-1 protein ($10 \mu\text{g kg}^{-1}$; R&D Systems, Minneapolis, MN), respectively, on a daily basis for 5 consecutive days. Bacteria genomic DNA was extracted from live *E. faecalis* as previously described,⁴⁸ and prepared in endotoxin-free water. Oral intubation for intragastric administration of the LTA (L2515, Sigma-Aldrich, $170 \mu\text{g kg}^{-1} \text{ day}^{-1}$) or the bacteria DNA ($165 \mu\text{g kg}^{-1} \text{ day}^{-1}$, 8.5×10^{10} equivalent $\text{CFU kg}^{-1} \text{ day}^{-1}$) was performed a day before virus infection.

Viral load determination. Tissues were harvested and homogenized using a mechanical homogenizer in serum-free DMEM. At the indicated time points post infection, PFU were determined in viral supernatants using the plaque assay in MDCK cells.

Quantification of leukocytes in BAL. Mice were anesthetized with an inhaled isoflurane overdose, and BAL samples were collected as previously described.⁴⁹ BAL cell content was determined using a hemocytometer in a blinded manner. BAL was then transferred to a cytocentrifuge sample container and cell smears for the differential leukocyte count (200 cells per specimen) were prepared.

Cytokine measurement. Samples were collected immediately after killing the mice and stored at -70°C until analysis. BAL, serum, and tissue cytokine levels were measured using commercially available immunoassays (Duoset ELISA Development Kits; R&D Systems) according to the manufacturer's protocol. All measurements were performed in triplicate. The intra- and inter-assay coefficients of variation were less than 15%.

Isolation of peritoneal macrophages for the *in vitro* virus challenge. The peritoneal macrophages were collected by a previously described procedure,⁵⁰ mice were intraperitoneally injected with 2 ml of 4% Brewer thioglycollate medium (Sigma-Aldrich) to increase the number of peritoneal macrophages. Peritoneal macrophages were harvested 4 days thereafter by peritoneal lavage in RPMI-1640 medium (Gibco, NY) and enriched by plastic adherence. Macrophages were cultured overnight in RPMI-1640 medium containing 5% heat-inactivated FBS (Hyclone, Logan, UT). Cells were then infected with different doses of viruses (multiplicity of infection of 5 and 10) for the indicated times. Negative uninfected controls were included in all experiments.

RNA extraction and quantitative real-time PCR. Total RNA was extracted from peritoneal macrophages using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. Real-time PCR was performed with the KAPA SYBR FAST qPCR Kits (Kapa Biosystems, Woburn, MA) for specimens and diluted standards. Serial dilutions of purified DNA were used for real-time PCR calibration. PCR-grade water was used as a negative control. Primer sequences used for amplifications were as follows: mouse GAPDH, 5'-CATGGCCTTCCGTGTTC-3' (forward) and 5'-CCTG GTCCTCAGTGTAGC-3' (reverse); mouse MCP-1, 5'-TGAAGCCA GTCTCTCTCTCC-3' (forward) and 5'-TGAGTAGCAGCAGGTGA GTG-3' (reverse). Experiments were performed in triplicate, with the coefficients of variation being $<5\%$. MCP-1 expression was normalized against GAPDH expression. The mean MCP-1 expression in *E. faecalis*-treated mice was subsequently normalized against that of the control group.

Clinical scoring method for enterovirus infections. Clinical scores were determined daily after EV71 infection as previously described.²⁷ The clinical scoring system was as follows: 0, healthy; 1, ruffled hair and hind limb weakness; 2, paralysis in one of the hind limbs; 3, paralysis in both of the hind limbs and fast movements; 4, paralysis in the hind limbs and forelimbs and being unable to move; and 5, death.

Statistical analysis. Data are expressed as means $\pm$ standard errors of the mean (s.e.m.). Survival curves were compared using the Gehan-Breslow-Wilcoxon test. Body weight values, clinical scores, and viral titers were analyzed using two-way repeated-measures ANOVA followed by the Tukey's *post-hoc* test. The Student's *t*-test was used to compare BAL cell counts and cytokine levels. All calculations were performed using the GraphPad Prism statistical software (GraphPad, San Diego, CA). Two-tailed *P* values <0.05 were considered as statistically significant.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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AUTHOR CONTRIBUTIONS

M.-F.C. and S.-R.S. conceived and designed the study; M.-F.C. performed most of the experiments; S.-Y.H., Y.-C.L., and S.-N.T. performed part of the experiments; K.-F.W. was in charge of the clinical scoring; M.-F.C., D.M.O., and S.-R.S. drafted the paper. All authors read and approved the final manuscript.

DISCLOSURE

The authors declared no conflict of interest.

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