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Heat-killed probiotic regulates the body’s regulatory immunity to attenuate subsequent experimental autoimmune arthritis

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ABSTRACT

Administration of inactivated probiotics has been proved to enhance host immunity. Herein, we aim to explore their potential in modulating systemic autoimmune disorders. The bovine type II collagen (CII)-induced arthritis (CIA) and CII antibody-induced arthritis (CAIA) mice models were used in this study. Heat-killed *Lactobacillus reuteri* (h-*L. reuteri*) was administered before or after the induction of CIA. The results indicated that the severity of CIA was alleviated and the prevalence of CIA was decreased in the mice pre-treated with h-*L. reuteri*. Using enzyme-linked immunosorbent assays, we found that decreased serum CII-specific IgG antibody IL-6 and CXCL1 but the increased level of IL-10 was found in h-*L. reuteri*-treated cohort. Moreover, h-*L. reuteri* treatment decreased the severity and incidence of arthritis in the CAIA model which was associated with a early decrease of IL-6. Systematic supplement of exogenous IL-6 reversed h-*L. reuteri*-induced CIA suppression. For regulatory immune responses, the frequency of Tregs and CD4 + IL-10 + cells was increased in the draining lymph of joint of h-*L. reuteri*-treated mice after second immunization. Parallelly, we found that if CIA was induced, CD103 + dendritic cells in mesenteric lymph nodes and α4β7 + Tregs in the spleen were increased in h-*L. reuteri*-treated mice, suggesting h-*L. reuteri* might affect the peripheral migration of Tregs to modulating CIA. Finally, the mice with progressive CIA were treated with h-*L. reuteri* after the second immunization. No alleviation of CIA severity, as well as an increase of splenic α4β7 + Tregs, was observed in these mice. This study indicates that pre-administration of h-*L. reuteri* can alleviate the CIA in mice and may serve as a promising strategy for autoimmune disease prevention.

1. Introduction

Rheumatoid arthritis (RA) is one of the common inflammatory arthritis diseases, which is characterized as progressive joint and synovium inflammation, leading to the destruction of the bone structures [1]. An epidemiologic study demonstrates that RA affects ∼1% human population, especially women [2]. Both genetic background and postnatal environmental effects can affect the risk for RA development [2]. Several specific immune-targeted therapeutics, such as TNF or IL-6 inhibition with antibodies, have been used for RA patients [3]. However, the new strategy for RA prevention is urgently needed.

Probiotics exhibit multiple activities to improve host immunity against pathogenic bacteria infections or protecting mucosal barriers from extreme inflammation [4–6]. Recent evidence indicates that administration with probiotics can prevent or ameliorate *Clostridium difficile* infection in patients receiving the antibiotics treatment [7]. *Lactococcus lactis* reduces the severity of *Vibrio cholerae*-induced diarrheal in infant mice [8]. Alternatively, heat-killed probiotics may be safer than live probiotics for children use, as the immune system of children is immature. The immunomodulatory effects of heat-killed probiotics were observed for two decades [9]. Matsuzaki T’s study finds that oral feeding of ovalbumin-sensitized BALB/c mice with heat-killed *Lactobacillus casei* inhibits the production of immunoglobulin E in serum [10]. Consistent with observations in live probiotics treatments, heat-killed probiotics also show the effects to alleviate infection, allergy and inflammatory disorders [11–13]. Supplement with heat-killed probiotics, *Lactobacillus*, ameliorates intestinal injury induced by dextran sulfate sodium [14]. In addition to intestinal diseases, several other diseases, such as liver and heart fibrosis, and alcoholic liver disease, benefited from heat-killed probiotics administration [15,16]. Attractively, gastrostomy-fed infant rats with heat-killed *Lactobacillus rhamnosus* reduced lipopolysaccharide (LPS)-induced inflammation through suppression of pro-inflammatory mediators and induction of anti-inflammatory mediators systemically [17]. However, whether heat-killed

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probiotics are a potential adjuvant therapy for autoimmune disorders is still unexplored.

2. Methods and materials

2.1. Mice and bacterial culture

Six-eight weeks old, specific-pathogen-free, female DBA/1 J mice were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd.. The *L. reuteri* MM2-3 (ATCC Number: PTA 4659) was purchased from ATCC (USA). This bacteria was cultured in MRS broth at 37 °C in the aerobic condition for 36–48 h. The cells (OD_{600nm} = 0.8–1.2) were collected and the amount of colony-forming unit was calculated by plate counting. Then the cells were killed by a boiling water bath for 30 min. 5 ml of suspension (OD_{600nm} = 1.0) was collected and re-suspend in 400 μl sterile water for subsequent animal administration.

2.2. Animal experiment design

The animal experimental procedures were approved by the Ethics Committee of Guangdong Medical University. For the collagen II (CII)-induced arthritis mice model, mice were immunized with Complete Freund's adjuvant (CFA, Sigma-Aldrich) emulsified with 2 mg ml^{-1} bovine CII (Chondrex), followed by immunization with Incomplete Freund's adjuvant (IFA) emulsified with bovine CII at the skin of hip around the tail, as shown in Fig. 1A. The h-*L. reuteri* was gavaged orally for consecutive 14 days at day 7–21 (Figs. 1–5) or day 21–35 (Fig. 1 and 6). At least six mice were enrolled in each group. For CII antibodies-induced arthritis (CAIA) mice model, mice were intravenously injected with 1.5 mg Anti-CII 5-clone cocktail (Chondrex) at day 14 and LPS was intraperitoneally injected three days later to evoke arthritis according to manufacturer's introduction. The severity of disease in each limb was identified using a 0–4 score method. Briefly, 0, no swelling; 1, mild red and swelling; 2, moderate red and swelling; 3, obvious swelling and joint stiffness; 4, severe swelling and joint stiffness with erythema. All the limbs in each mice were calculated individually and pooled to acquire final score.

2.3. Serum antibody assessment

Serum was collected immediately after killing. For CII-specific IgG titers determination, ELISA plates were first coated with 0.1 mg ml^{-1} of CII at 4 °C for 16 h and then blocked by 10% fresh goat serum/phosphate buffered saline (PBS)/0.5% Tween 20 (Bioind) at 37 °C for 2 h. A serial dilution of serum samples (range 1,000–10,000,000) was added in triplicate and incubated at 37 °C for 2 h. After washing with PBS/0.5% Tween 20, the plates were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Santa Cruz) at 37 °C for 1 h. The plates were washed five times with PBS/0.5% Tween 20 and incubated with tetramethylbenzidine (TMB, Solarbio, China). If a slight blue coloring was observed in the control serum, the reaction was stopped by stop solution (Solarbio, China). The absorbance was obtained at 450 nm. The titers of IgG antibody were identified as the dilution that OD value was 2-fold higher than that of the negative control.

2.4. Serum cytokine assessment

Commercial ELISA kits were purchased from Dakewe Biotech Co., Ltd (Shenzhen, China) or PeproTech Inc. (USA). Serum IL-6, IL-10 and CXCL1 (KC) levels were measured according to the introductions of the manufacturer.

2.5. Flow cytometric analysis (FACS)

To prepare single cell-suspension, the inguinal lymph nodes (ILN), mesenteric lymph nodes (MLN) and spleen were mechanically dispersed, followed by filtering through a 70-μm cell strainer. After washing with PBS, the cells were re-suspended in FACS buffer for antibody staining or in RPMI 1640 containing 10% heat-inactivated fetal bovine serum. For Tregs or α4β7 + Tregs determination, the cells were first stained with extracellular markers and then fixed and permeabilized with Mouse Foxp3 Buffer Set (BD Pharmingen). For CD4 + IL-10 + cells determination, cells were stimulated with 500 ng ml^{-1} phorbol-12-myristate 13-acetate and 500 ng ml^{-1} ionomycin in the presence of 5 μg ml^{-1} brefeldin A. After staining with extracellular markers, the cells were fixed and permeabilized, and then stained with IL-10 antibody. The following antibodies were used in these experiments: CD45 (30-F11), CD4 (RM4-5), CD25 (3C7), Foxp3 (MF-14), IL-10 (JES5-16E3), CD11c (N418), MHCII (M5/114.15.2), CD103 (2E7), α4β7 (DATK32). All antibodies were purchased from eBioscience. The flow cytometry analysis was performed on a BD Calibur Flow Cytometer.

2.6. Statistical analysis

Data were presented as means and standard errors of the mean (SEM). All analysis was performed with the GraphPad Prism software (GraphPad, San Diego, CA). The Mann–Whitney U-test was used for the comparison between two groups. $p < 0.05$ was considered as the statistically significant difference.

3. Results

3.1. Treating CIA mice with heat-killed Lactobacillus reuteri reduced the severity of arthritis

To investigate whether oral administration of heat-killed *L. reuteri* could suppress the severity of bovine type II collagen (CII)-induced autoimmune arthritis (CIA), we used DBA/1 J mice to induce CIA and the *L. reuteri* MM2-3, a stain that has been confirmed for reducing colitis, for treatment [6]. The DBA/1 J mice were divided into three groups: normal control (without CIA), saline (vehicle) or heat-killed *L. reuteri* (h-*L. reuteri*). This treatment started at 7 days post first immunization and performed daily for 14 days (Fig. 1A). Once the second immunization was performed, we followed and compared the clinical symptoms of CIA between vehicle and h-*L. reuteri* groups. The results found a comparably decreased severity of arthritis and a reduction of CIA incidence (12/18) in h-*L. reuteri*-treated cohort (Fig. 1B-C). At day 34, most mice treated with vehicle showed severe CIA symptoms (Fig. 1D, i.e, inflammatory cell infiltration (upper) and bone destruction (bottom)); in comparison, the arthritis scores in h-*L. reuteri*-treated mice were lower and converted to decline in the following days. Of note, 6 of 18 mice in h-*L. reuteri*-treated cohort did not emerge CIA symptoms.

3.2. Antibody and cytokine production in the serum of heat-killed Lactobacillus reuteri-treated mice

We next analyzed the serum CII-specific IgG antibody, IL-6, IL-10 and CXCL1 levels by ELISA. Treating mice with h-*L. reuteri* significantly reduced the serum CII-specific IgG antibody production (Fig. 2A). The mice that failed to develop CIA in h-*L. reuteri*-treated cohort showed a further low level of serum CII-specific IgG antibody. Detection of serum IL-6 and IL-10 indicated that IL-6 was reduced but IL-10 was increased in the h-*L. reuteri*-treated cohort (Fig. 2B and C), suggesting the attenuated inflammatory response and vigorous regulatory response. Of note, IL-10 production was remarkably increased in mice receiving h-*L. reuteri*-treatment but did not develop CIA. For serum CXCL1, the CXCL1 levels were significantly decreased in the h-*L. reuteri*-treated cohort (Fig. 2D). Therefore, oral administration of mice with h-*L. reuteri* might enhance the regulatory response and inhibit the production of CIA inducers to suppress the severity of CIA.

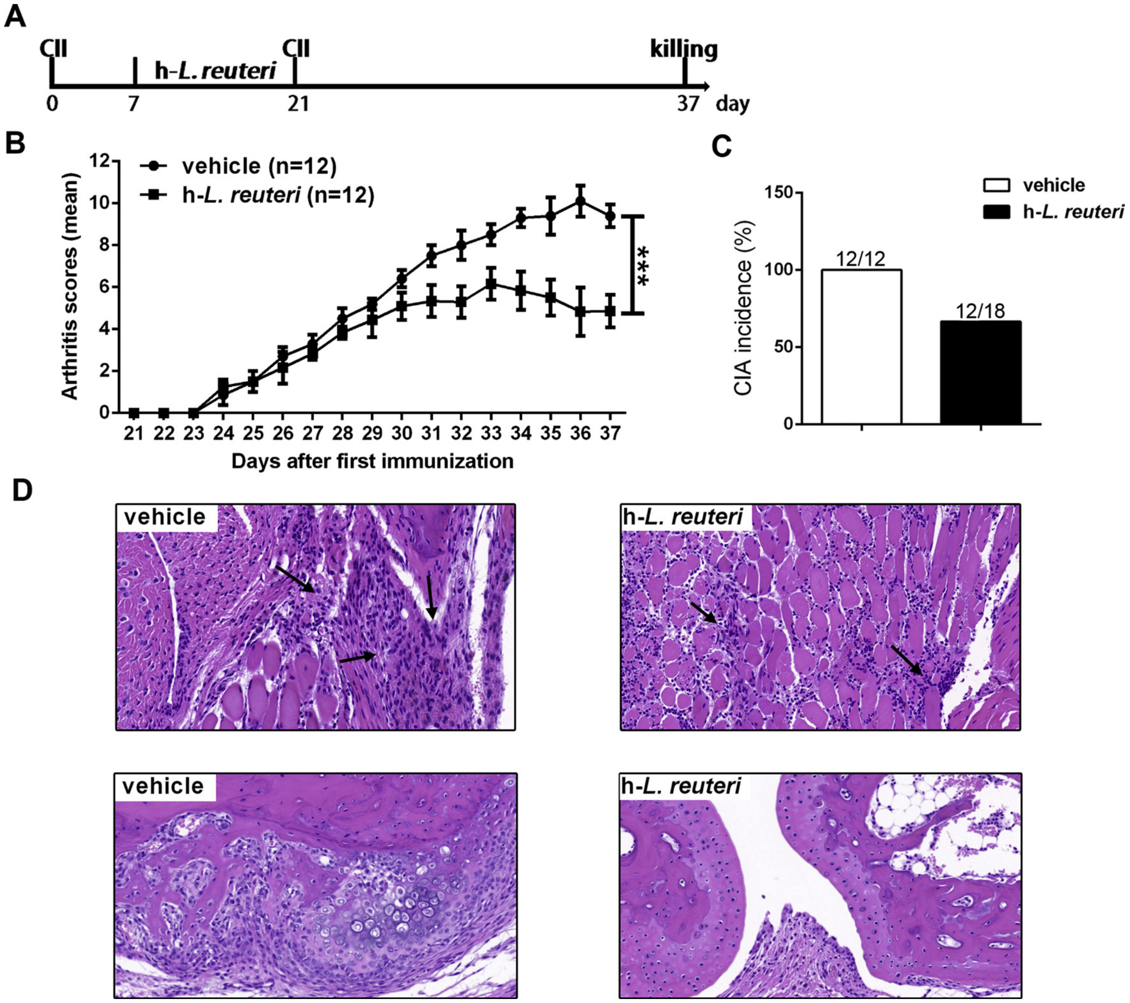

Fig. 1. Oral administration of mice with h-*L. reuteri* before second immunization alleviated the severity of CIA. (A) Schematic representation of animal administration. Saline (vehicle) and 5×10^9 CFU h-*L. reuteri* were orally administrated with CIA mice for 14 consecutive days before second immunization. (B) The mean scores of CIA were measured daily after the second immunization. Only the mice that developed CIA were involved. (C) The incidence of CIA in each groups. (D) Representative images of H&E staining. Upper, the inflammatory areas. Bottom, the alteration of cartilage. $***p < 0.001$ ($n = 12$ mice per group, the Mann–Whitney U-test).

3.3. Heat-killed Lactobacillus reuteri treatment impacted the progression of arthritis through modulation of serum CII-specific IgG and IL-6 production

To query whether h-*L. reuteri* treatment impacted CII-specific humoral immune response to suppress experimental arthritis, we utilized a mice model that induced arthritis by injection of CII-specific antibodies. Mice were treated with h-*L. reuteri* or vehicle during day 0–14 and 1.5 mg anti-CII 5-clone antibody cocktail was intravenously administered at day 14. 50 μg LPS was intraperitoneally injected at three days later to induce CII antibody-induced arthritis (CAIA) (Fig. 3A). The results showed that mice treated with h-*L. reuteri* displayed a lower degree of arthritis scores as compared with mice treated with vehicle during day 17–23, although subsequently the difference was narrowed (Fig. 3B). The prevalence of CAIA was also decreased in h-*L. reuteri* treated mice, as the incidence rate on vehicle and h-*L. reuteri* cohorts was 100% (8/8) and 75% (6/8), respectively (Fig. 3C). Moreover, we measured serum IL-6 levels in all CAIA mice on day 22 and day 27. The results suggested that IL-6 levels in h-*L. reuteri* cohort (CAIA) was lower than that in vehicle cohort at day 22 but not day 27 (Fig. 3D), implicating that h-*L. reuteri* treatment suppressed the development of arthritis through reducing systemic IL-6 production. In other words, when CII-specific antibody level was equivalent, IL-6 production might accelerate the development of experimental arthritis. This results implicated a potential antibody-independent regulatory response in h-*L. reuteri* cohort. Parallelly, to investigate the role of IL-6 on CIA development after h-*L. reuteri* treatment, mice were intraperitoneally injected with three doses of 1 μg IL-6 on day 22, 23 and 24 (Fig. 4E). The development of CIA was accelerated in mice receiving extraneous IL-6, although h-*L. reuteri* has been administrated (Fig. 4F). In addition, all the mice receiving IL-6 developed CIA during day 22–24, but the incidence rate of saline cohorts at the final of this experiment was 71.4% (10/14) (Fig. 4G). A higher level of CII-specific IgG antibody was also found in the recipients of IL-6 as compared with that receiving saline (Fig. 4H). No significant difference was observed between h-*L. reuteri*-treated mice receiving saline or IL-6. Collectively, these results indicated that h-*L. reuteri* treatment might suppress CIA development through modulation of CII-specific IgG antibody and early IL-6 production.

3.4. Regulatory T cells and IL-10-producing CD4 + T cells were increased in heat-killed Lactobacillus reuteri-treated mice post second immunization

To investigate the regulatory immune response in these mice, we measured Tregs and CD4 + IL-10 + cells in inguinal lymph node (ILN) 10 days after the h-*L. reuteri* treatment. Without CIA induction, oral administration of mice with h-*L. reuteri* alone did not induce the

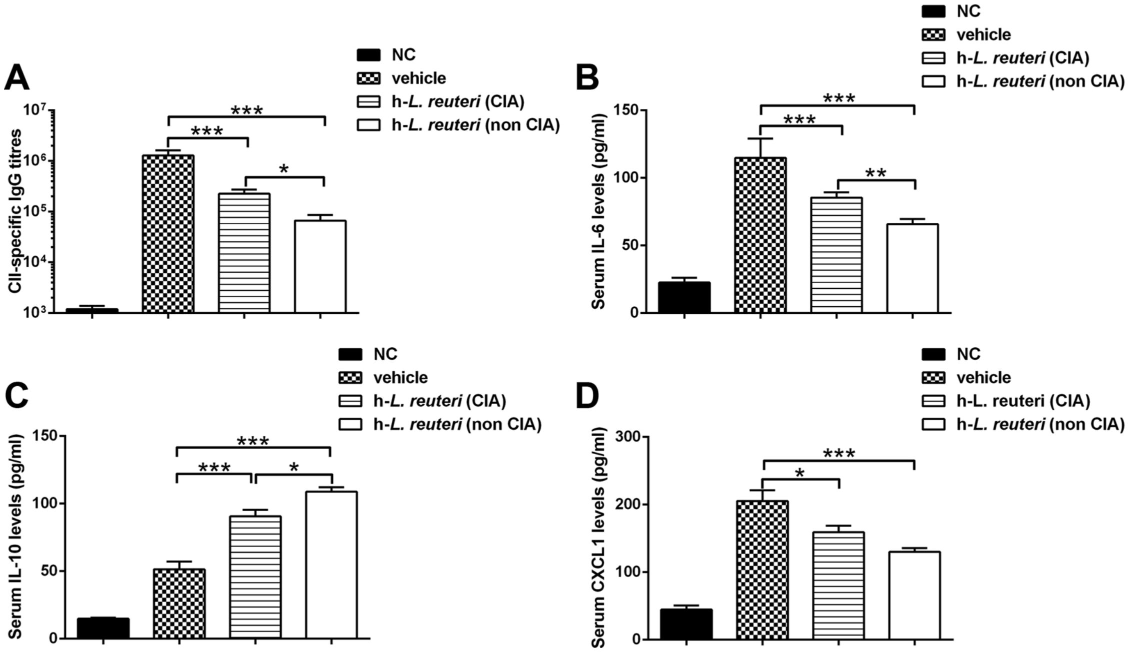

Fig. 2. Antibody and cytokine production in serum of h-*L. reuteri* -treated mice. Serum was obtained at day 37 from the following groups: naïve control (NC), vehicle-treated mice, h-*L. reuteri* -treated mice with or without CIA. CII-specific IgG titres (A), serum IL-6 levels (B), serum IL-10 (C), and serum CXCL1 (D) in CIA mice receiving vehicle and h-*L. reuteri* at day 37. *p < 0.05, **p < 0.01, ***p < 0.001 (n = 6–12 mice per group, the Mann–Whitney U-test).

expansion of Tregs in ILN (Fig. 4A and B). However, the frequency of Tregs (CD25 + Foxp3 +) on CD4 + T cells was significantly increased in CIA mice. As compared with vehicle group, more Tregs were observed in the ILN of h-*L. reuteri*-treated mice (Fig. 4C and D). Additionally, IL-10-producing CD4 + T cells were also increased in h-*L. reuteri*-treated mice. These data suggested that h-*L. reuteri*-treated mice demonstrated a strong regulatory immune response post CIA induction.

3.5. CD103+ dendritic cells were augmented in mesenteric lymph nodes and promoted the egress of regulatory T cells

As mice were administered with h-*L. reuteri* orally, we speculated that regulatory immune cells from gut might contribute to the enhanced systemic regulatory immune response. The results of FACS assays showed no alteration of Tregs and CD4 + IL-10 + T cells in mesenteric lymph nodes (MLN) of mice with or without CIA response (Fig. 5A and B). CD103 + dendritic cells (DCs) in MLN were derived from the lamina

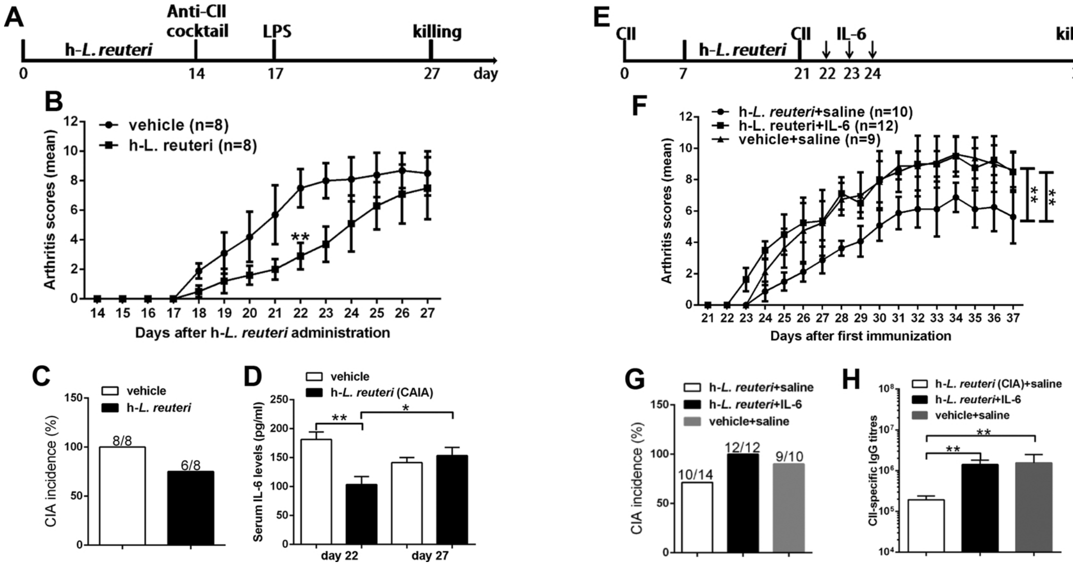

Fig. 3. Heat-killed *Lactobacillus reuteri* treatment impacted the progression of arthritis through modulation of serum CII-specific IgG and IL-6 production. (A) Schematic representation of animal administration. Mice were treated with h-*L. reuteri* or vehicle during day 0–14 and 1.5 mg anti-CII 5-clone antibody cocktail was intravenously administered at day 14. 50 μg LPS was intraperitoneally injected at three days later. (B) The mean scores of CIA were measured daily after the LPS injection. (C) The incidence of CIA were summarized. (D) Serum IL-6 level was measured on day 22 and day 27. *p < 0.05, **p < 0.01 (n = 6–8 mice per group, the Mann–Whitney U-test) (E) Schematic representation of animal administration. IL-6 or saline was intraperitoneally administrated with h-*L. reuteri*-treated CIA mice for three days from day 22–24 after second immunization. (F) The mean scores of CIA were measured daily after the second immunization. Only the mice that developed CIA were involved. (G) The incidence of CIA were summarized. (H) CII-specific IgG titres on day 37 was measured by ELISA. Mean CIA scores in all cohorts were calculated in mice with CIA. *p < 0.05, **p < 0.01, ***p < 0.001 (n = 10–14 mice per group, the Mann–Whitney U-test).

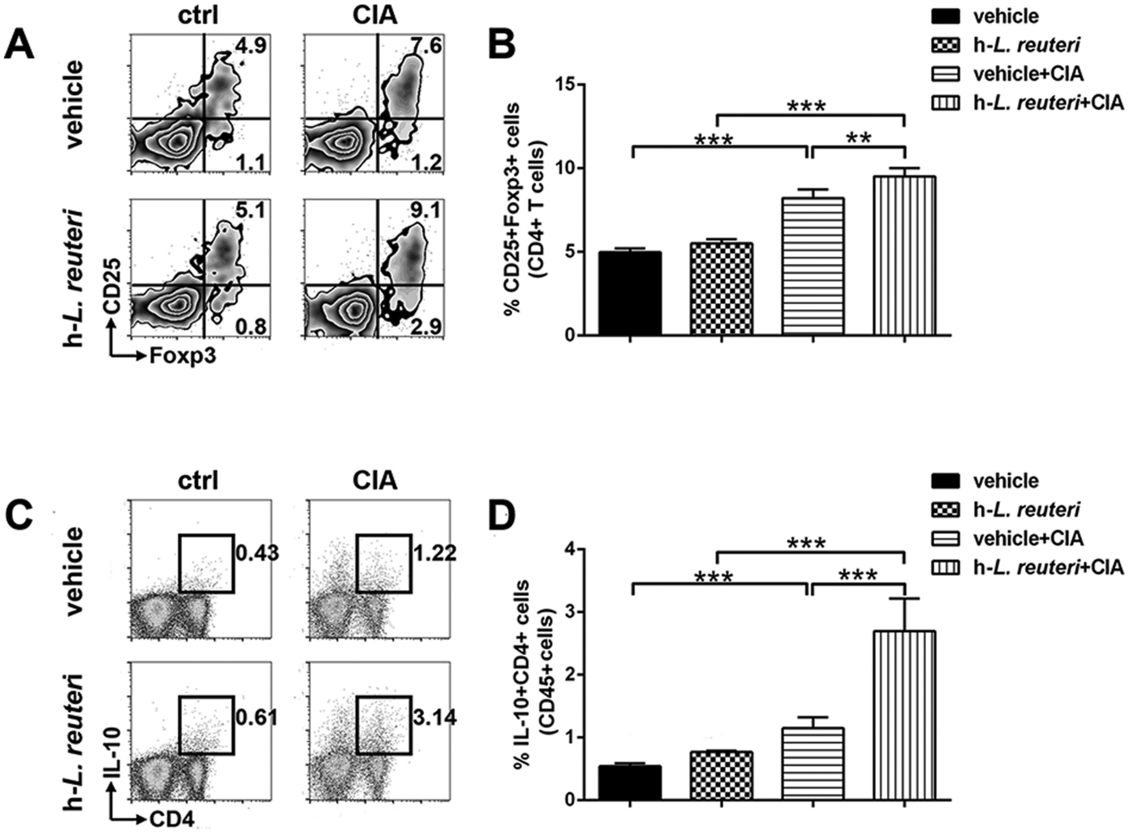

Fig. 4. Regulatory T cells and IL-10-producing CD4 + T cells were increased in heat-killed *Lactobacillus reuteri*-treated mice post second immunization. Saline (vehicle) and h-*L. reuteri* were orally administrated to CIA or normal mice for 14 consecutive days before the day of second immunization according to Fig. 1A. All mice were sacrificed at day 30. Representative measurements of Tregs on CD4+ cells (A) or CD4 + IL-10 + T cells on CD45+ cells (C). The frequency of Tregs on CD4+ cells (B) and CD4 + IL-10 + T cells on CD45+ cells (D) in inguinal lymph nodes was measured by FACS. **p $<$ 0.01, ***p $<$ 0.001 (n = 5–6 mice pre group, the Mann–Whitney U-test).

propria DCs of the gut, which cooperated with retinoic acid and cytokine TGF-β to facilitate the generation of gut-homing Tregs. Thus, we measured the CD103+ DCs of MLN in h-*L. reuteri*-treated mice. The frequency of CD103 + DCs on total DCs was significantly increased in the h-*L. reuteri*-treated mice after CIA emergence (Fig. 5C and D). Consistent with these results, α4β7 + Tregs in spleen were also significantly augmented (Fig. 5E and F), indicating h-*L. reuteri* might affect gut DCs to alter peripheral migration of MLN Tregs during CIA response.

3.6. *Treating CIA mice with heat-killed Lactobacillus reuteri after second immunization failed to reduce arthritic pathology*

Clinical evidence with small sample size suggested that *L. reuteri* treatment did not improve the symptoms of RA patients [18]. Hence, we measured the effect of h-*L. reuteri* treatment when CIA have been induced. The h-*L. reuteri* administration was performed after the second immunization (Fig. 6A). As shown in Fig. 6B and E, the results of arthritis scores showed no difference between vehicle and h-*L. reuteri* groups. In addition, IL-10 in serum and α4β7 + Tregs in spleen were not elevated (Fig. 6C and D). Hence, it seemed that administrating h-*L. reuteri* before the CIA emergence was critical for the generation of the systemic regulatory immune response.

4. Discussion

In this study, our results showed that heat-killed probiotics could relieve the severity of following autoimmune arthritis through promoting the peripheral migration of gut-derived Tregs, but showed little effect for treating progressive CIA. A previous study using *Lactobacillus helveticus* for CIA prevention obtained a similar result that oral administration of *Lactobacillus helveticus* suppressed the CIA symptoms through reducing the abundance of immune cells in draining lymph node, and several indicators of CIA, CII-specific antibodies and IL-6, were decreased [19]. The results of the present study suggested that in CIA and CAIA model, h-*L. reuteri* modulated the IL-6 production which was critical for CIA development. Increased anti-inflammatory cytokine IL-10 level indicated a strong regulatory immune response in the h-*L. reuteri*-treated mice. Two critical regulatory subsets of CD4 + T cells (Tregs and CD4 + IL-10 + T cells) were increased in the draining lymph node post CIA emergence. Without induction of CIA, no increase of these cells could be detected. Thus, it seemed like that oral administration with h-*L. reuteri* shaped the host immune system to repress the autoimmune response.

Gut immune homeostasis affects the outcome of peripheral immunity [20]. When h-*L. reuteri* was administered orally, their degradation products (including nucleic acid and cell wall polysaccharides) or whole cells could activate epithelial cells or DCs by pattern recognition receptors, such as Toll-like receptors (TLRs) [21,22]. TLR2 and TLR9 might be involved in the h-*L. reuteri*-mediated tolerant response during CIA. A critical study indicated that gut commensal *Bacteroides fragilis* utilized host TLR2 pathway to drive regulatory immune response, and established host-microbial symbiosis [23]. The polysaccharide A of *Bacteroides fragilis* was recognized as a vital constituent for establishing immunologic tolerance [24]. Mice lacking TLR9 showed the high frequency of Tregs and less function of effector response against intestinal infection [22]. As microbial DNA was a major agonist for TLR9, the DNA from probiotics contained much

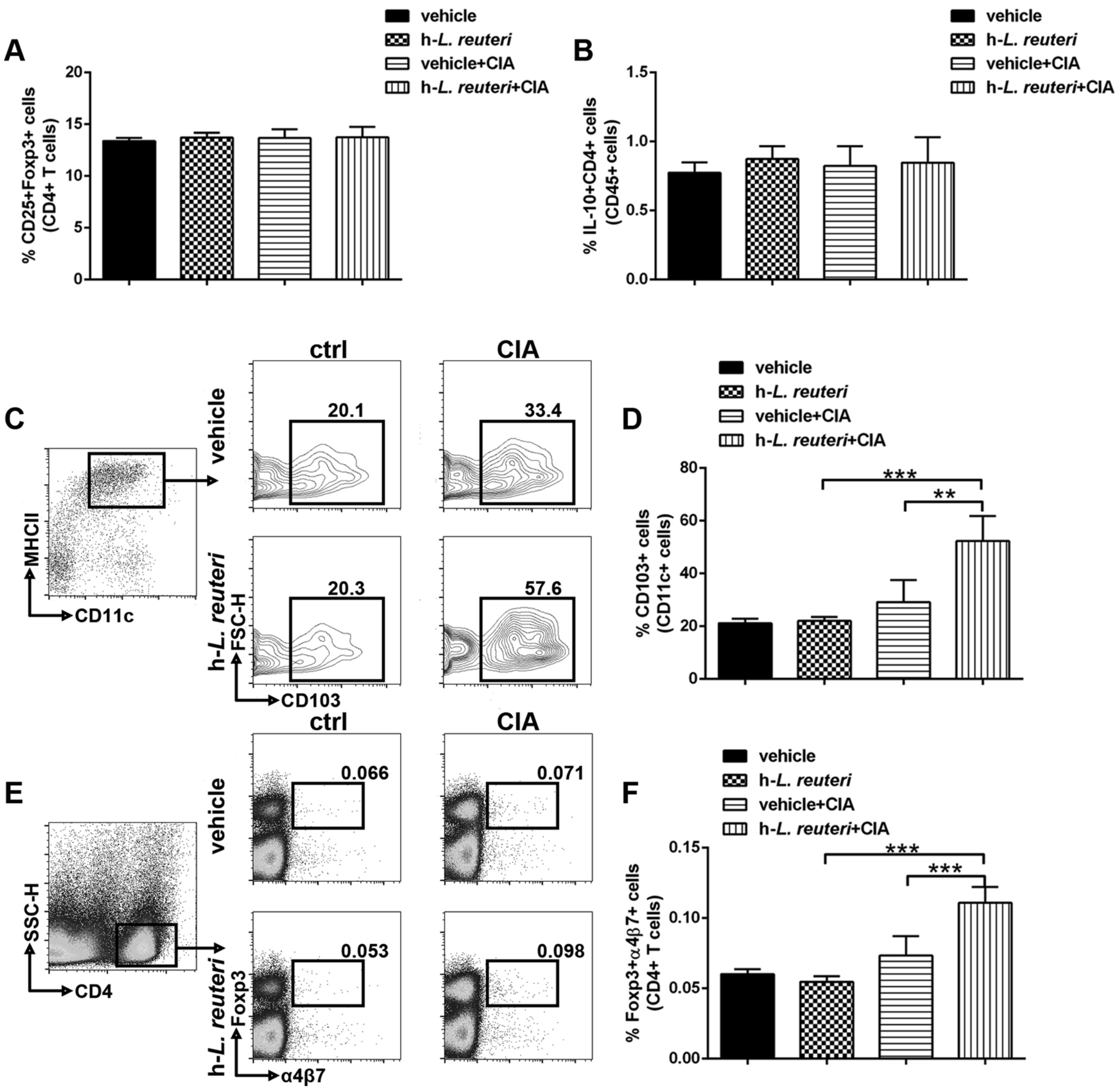

Fig. 5. CD103+ dendritic cells were augmented in mesenteric lymph nodes and promoted the egress of regulatory T cells. Mice were administrated as above. The frequency of Tregs on CD4+ cells (A) and CD4+IL-10+ T cells on CD45+ cells (B) in MLN was measured by FACS. Representative measurements of CD103+ cells on CD11c + MHCII + cells (C) in MLN or Foxp3+α4β7+ cells on CD4+ cells (E) in spleen. The frequency of CD103+ cells on CD11c + MHCII + cells (D) in MLN and Foxp3+α4β7+ cells on CD4+ cells (F) in spleen was measured by FACS. **$p < 0.01$, ***$p < 0.001$ ($n = 5–6$ mice per group, the Mann–Whitney U-test).

more suppressive motif, which induced the host tolerant immunity; in comparison, DNA from pathobionts showed the high frequency of cytosine phosphate guanosine (CpG) [25,26]. This notion was further confirmed by Wu et al's study using a K/BxN serum transfer mice model that both preventive or therapeutic administration of certain immunosuppressive CpG-ODNs inhibited the development of inflammatory arthritis [27]. More recently, emerging evidence highlighted the endogenous DNA/TLR9 pathway in inducing T cell-dependent autoimmune arthritis, as streptococcal cell wall (SCW)-induced arthritis in TLR9 knockout mice was repressed [28]. Here, we speculated that the constituents of h-*L. reuteri* might modify host immunity for CIA through the activation of tolerant immune response. Another observation of the present study was that in MLN, h-*L. reuteri* promoted the peripheral migration of Tregs through induction of CD103+DCs. MLN CD103+DCs migrated from the lamina propria of gut after antigen encounter [29]. In MLN, they cooperated with retinoic acid and other cytokines to induce gut-homing Tregs. After CIA emergence, the Tregs were augmented in ILN and at the same time, an increase of α4β7+ Tregs was observed in spleen, a central immune organ. These data suggested that h-*L. reuteri* might enhance the peripheral regulatory immune response by changing the tolerance immunity in the intestine. Also, in the focus of arthritis, the relationship between TLRs and chronic inflammation should not be ignored neither. Recent review proposed self-sustaining chronic inflammatory loop maintained through Toll-like receptor (TLR) activation. [30] Therefore, the question raised, as oral administration of h-*L. reuteri* might affect the operation of this loop to inhibit the development of CIA.

Others immune routes, such as intraperitoneally and subcutaneously, were also used for heat-killed probiotic administration. Intraperitoneal administration of mice with *Lactobacillus helveticus* completely protected mice from CIA [19]. The mechanism might be associated with decreased germinal center B cells, and CD4 + T cells in draining lymph nodes, but why intraperitoneal administration induced such powerful effect to inhibit the CIA needed more explorations. Another study found that subcutaneous administration of *Lactobacillus salivarius* alleviated both colitis and CIA in experimental mice [31]. So there will be interested to study whether heat-killed probiotic treatment is effective in other administration routes. However, invasive injection of heat-killed probiotic might confront more problems for clinic use than oral administration, because of safety. In the consideration of further clinic practice, we selected oral route but not invasive routes.

The dosage regimen of probiotic administration is responsible for

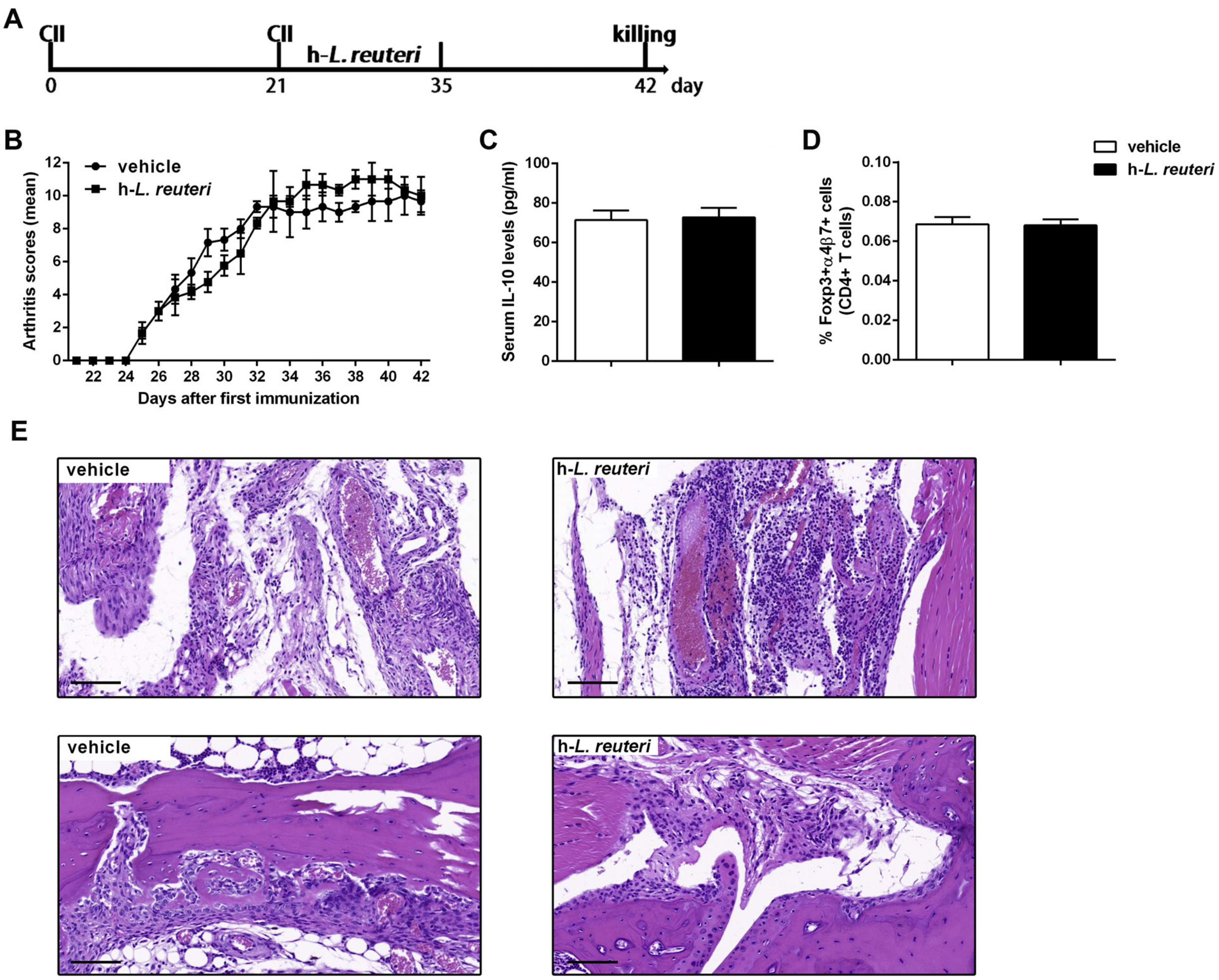

Fig. 6. Treating CIA mice with heat-killed *Lactobacillus reuteri* after second immunization failed to reduce arthritic pathology. (A) Schematic representation of animal administration. Saline (vehicle) and 5 × 10^9 CFU h-*L. reuteri* were orally administrated with CIA mice for 14 consecutive days after second immunization. (B) The mean scores of CIA were measured daily after the second immunization. Serum IL-10 (C) level and the frequency of Foxp3 + α4β7 + cells on CD4 + cells (D) in spleen were measured. (E) Representative images of H&E staining. Upper, the inflammatory areas. Bottom, the alteration of cartilage. (n = 6 mice per group, the Mann–Whitney U-test).

the subsequent regulatory immune response. In this study, oral administration of h-*L. reuteri* before second immunization suppressed the severity of CIA; however, administration of h-*L. reuteri* after CIA emergence failed to repress CIA. Similar results were observed in a virus-infected mice model [32]. Possibly, the healthy host might be more sensitive to h-*L. reuteri* that altered immune homeostasis after administration, while the individuals that underwent an acute inflammatory response were insensitive to this stimulation. Moreover, the composition of gut microorganisms in RA patients was distinct from that of the healthy subject, indicating a potential influence of gut microorganisms [33]. Of note, this view has been proved in mice by Liu et al.'s study [34].

The present study found that oral administration of h-*L. reuteri* could suppress the severity of CIA in mice and related to an expansion of regulatory immune response. h-*L. reuteri* induced CD103 + DCs, which then triggered the peripheral migration of Tregs to enhance regulatory immunity after CIA emergence. This study provided insight into the mechanism of oral administration of heat-killed probiotic for autoimmune disease prevention.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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