Delivery of IL-35 by *Lactococcus lactis* Ameliorates Collagen-Induced Arthritis in Mice

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IL-35, a relatively newly discovered cytokine belonging to the larger IL-12 family, shows unique anti-inflammatory properties, believed to be associated with dedicated receptors and signaling pathways. IL-35 plays a pivotal role in the development and the function of both regulatory B (Bregs) and T cells (Tregs). In order to further its therapeutic potential, a dairy *Lactococcus lactis* strain was engineered to express murine IL-35 (LL-IL35), and this recombinant strain was applied to suppress collagen-induced arthritis (CIA). Oral administration of LL-IL35 effectively reduced the incidence and disease severity of CIA. When administered therapeutically, LL-IL35 abruptly halted CIA progression with no increase in disease severity by reducing neutrophil influx into the joints. LL-IL35 treatment reduced IFN-γ and IL-17 3.7- and 8.5-fold, respectively, and increased IL-10 production compared to diseased mice. Foxp3$^+$ and Foxp3$^-$ CD39$^+$ CD4$^+$ T cells were previously shown to be the Tregs responsible for conferring protection against CIA. Inquiry into their induction revealed that both CCR6$^+$ and CCR6$^-$ Foxp3^{+or-} CD39$^+$ CD4$^+$ T cells act as the source of the IL-10 induced by LL-IL35. Thus, this study demonstrates the feasibility and benefits of engineered probiotics for treating autoimmune diseases.

Keywords: *Lactococcus*, probiotic, IL-35, therapeutic, IL-10, cytokines, regulatory T cells, CCR6

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, inflammatory, systemic autoimmune disease that affects about 0.24% of the worldwide population, and roughly 1% of the general population in Western countries. RA is two to three times more common in women than in men (1–5). RA manifests as a chronic synovitis and progressive destruction of the joints, leukocyte infiltrates, and cartilage destruction and bone erosion. Approximately half of the afflicted patients become disabled over the progression of this disease (6). RA is mediated predominantly by CD4$^+$ T cells overexpressing proinflammatory cytokines, particularly in the joints (7).

In order to test the efficacy of potential RA therapeutics and understand mechanisms of disease, the collagen-induced arthritis (CIA) model is often used (8). CIA is typically induced by immunizing rodents with bovine or chick type II collagen together with an adjuvant. This combination leads to immune attack of the host's native collagen involving components of both the innate and adaptive immune systems. Emphasis on regulating proinflammatory cytokines, particularly TNF-α, is key to minimizing disease since TNF-α can be detected in joints of RA patients (9, 10). Mouse CIA shares several clinical, histopathological and immunological features with human RA. Clinical features include erythema, edema, synovitis, pannus formation, and

CD4^{+} T cell-mediated inflammation with extensive cartilage and bone damage, resulting in joint deformities (11–13). These similarities are commonly exploited to use CIA as a model for RA and as a tool to investigate novel approaches to prevent and treat RA. Current treatments focus on neutralizing TNF-α action via anti-TNF-α mAbs and TNF-α receptor antagonists (14, 15); however, such interventions have been problematic, making patients more susceptible to opportunistic infections (14–16). Hence, alternatives that can restore tolerance need to be sought.

In view of reducing autoimmunity, the use of probiotics can restore immune homeostasis to reduce autoimmunity (17–19). Historically, lactic acid bacteria (LAB) represented the core of probiotic-based interventions, although more recently nonpathogenic *E. coli* (20–22), attenuated *Salmonella* (23, 24), *Bifidobacterium spp.* (25), and some yeasts like *Saccharomyces boulardii* (26) also proved to be valuable tools as novel therapeutic and prophylactic interventions. Traditional molecular genetics, coupled with synthetic biology, provides an ample selection of promoters and terminators resulting in dynamic expression levels. Protein synthesis can be induced *in vitro* under nisin controlled expression (NICE), or use a promoter that is silent during *in vitro* culture, and only active *in vivo* subsequent infection of the host (27–29). LABs are considered ideal vectors for oral or mucosal delivery since they are inherently nonpathogenic, and they can survive the harsh conditions of the gastric environment. LABs are amenable to recombinant expression of passenger antigens (Ags) to stimulate immunity against a number of pathogens (30–32), to curb the effects of inflammatory bowel disease (33, 34), to control the proliferation of cancer cells (35), and to use for enzyme replacement therapy (36) among other applications (27, 37, 38). Currently, the only microbiota-based therapy that is FDA-approved and commercially available is fecal microbiota transplant (FMT) to treat *Clostridium difficile* infections. However, close to 200 microbiome-based therapeutics and diagnostics are currently in development (39).

The delivery of oral therapeutics represents a significant advantage of adapting LABs. In this context, we developed recombinant *Lactococcus lactis* (LL) for oral delivery to treat autoimmune disease (40). In a similar fashion, the studies described here focus on the expression of the immunosuppressive cytokine, IL-35. Oral administration of probiotic-based therapeutics is considered ideal because the gastrointestinal (GI) tract is home to T cells that can be stimulated to become Tregs and to seed other mucosal and systemic immune compartments. Another advantage of using genetically-modified (GM) probiotics is that these have been shown to be both effective and safe (37, 41–43). Our previous work has shown that an engineered LL derived from an industrial dairy strain can ferment commercial milk to a yogurt-like product, and when applied for treatment of CIA, can maintain the same therapeutic properties as when grown on a synthetic medium (40). IL-35 belongs to the IL-12 cytokine family [rev. in (44)]. This heterodimeric cytokine is composed of IL-12p35 and IL-27EBI3 and, in contrast to most members of the IL-12 family, has potent anti-inflammatory attributes. This property is mediated via IL-35

binding both IL-12Rβ2 chain and gp130, which results in specific triggering of STAT1 and STAT4 on T cells (45) and IL-12Rβ2 and IL-27Rα on B cells (46). IL-35 is immunosuppressive for a number of autoimmune disease models including CIA (47, 48), experimental autoimmune encephalomyelitis (49, 50), uveitis (46), type 1 diabetes (51), inflammatory bowel disease (IBD, and psoriasis (52).

CCR6 was previously shown to be expressed by Tregs (53), particularly those expressing RoRγt (54, 55). These Tregs have been shown involved in suppressing autoimmune diseases (56–58). CCR6^{+} Tregs have been found more commonly associated with human Tregs (55, 59), but CCR6 has also been found to be induced in mice subjected to CIA (55).

Given its potency to treat various autoimmune diseases (46–52), we queried the effectiveness of live vector delivery of IL-35. To accomplish this objective, murine IL-35 was expressed in *L. lactis* subsp lactis IL1403 (LL-IL35), and tested for its ability to ameliorate CIA. Results show that LL-IL35 is highly effective in treating CIA via the stimulation of CCR6^{+} and CCR6^{−} Tregs producing IL-10 and suppressing the proinflammatory cytokines, IL-17 and IFN-γ.

MATERIALS AND METHODS

Bacterial Strain Engineering and Maintenance

Lactococcus lactis subsp. lactis IL1403 (IL1403) was grown on M17 plus 0.5% glucose (M17G). Microbiology work was performed according to NIH guidelines. Initial attempts to express IL-35 under the control of the constitutive p23 promoter yielded only rearranged, nonfunctional clones which confirmed the notion that IL-35 is difficult to express and to stabilize in a wide panel of hosts [our unpublished observations; (44, 48)]. To express IL-35, a synthetic gene codon-optimized for LL was designed in-house and then synthesized by Genscript (Piscathawa, NJ). The fragment contains an optimal Shine-Dalgarno sequence properly spaced from the ATG start codon, the usp45 secretion signal, the p35 coding region, a short flexible linker, the EBI3 coding region, and AgeI and SmaI sites at both ends. The fragment was excised with AgeI, gel-purified and cloned into pMSP3535H3 (53; a kind gift of Dr. DA Mills, University of California, Davis) yielding a construct named pBzMM150 (LL-IL35). Expression was achieved under the control of the nisin-inducible promoter borne on the vector.

Collagen-Induced Arthritis (CIA)

All the animal experiments described in the present study were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal studies were conducted under protocols approved by Montana State University's and the University of Florida's Institutional Animal Care and Use Committee.

C57BL/6 males (B6; 8- to 10-weeks of age; Charles River Laboratories, Horsham, PA USA) were maintained at Montana State University Animal Resources Center or the University of Florida Animal Center Services. Groups of B6 males were

induced with CIA using 100 µg of chicken collagen II (CII; Chondrex, Redmond, WA USA) emulsified in complete Freund's adjuvant (CFA) and administered s.c. as previously described (48, 60, 61). To treat CIA, mice were first orally gavaged with sterile 50% saturated sodium bicarbonate solution to neutralize stomach acidity, followed by 5×10^8 CFUs of LL vector or LL-IL35, or vehicle only, sterile PBS. Two dosing regimens were tested, three doses administered on days 14, 21, and 28, and two doses given on days 18 and 25 post-CII challenge. Clinical scores were measured in a double-blind fashion after treatment, and mice were monitored to day 40. Each of the four limbs was evaluated using a scale of 0–3 (48, 60, 61): 0, no clinical signs; (1) mild redness of a paw or swelling of single digits; (2) significant swelling of ankle or wrist with erythema; (3) severe swelling and erythema of multiple joints; maximum score per mouse is 12.

Cytokine Elisa

$CD4^+$ T cells were cell-sorted by negative selection on magnetic beads (Invitrogen, Grand Island, NY USA) from axillary, popliteal, and inguinal lymph nodes (LNs) yielding purity >98%. Purified $CD4^+$ T cells (3×10^6/ml) were restimulated with 5 µg/ml plate-bound anti-CD3 mAb (eBioscience, San Diego, CA USA) plus 5 µg/ml of soluble anti-CD28 mAb (eBioscience) for 48–72 h at 37°C and 5% CO_2 similar to that previously described (48). Culture supernatants were collected for cytokine-specific ELISAs (48, 60, 61).

Flow Cytometry

Splenic and LN cells were stained with fluorochrome-labeled mAbs to CD4, CD39, Ly-6G, Ly-6C, CD11b, and Foxp3 (eBioscience, San Diego, CA USA), TGF-ß (R&D Systems, Minneapolis, MN USA), and fluorochrome-conjugated streptavidin (BD Pharmingen, San Jose, CA USA). For flow cytometry of Tregs, whole splenic and LN cells (5×10^6/ml culture) were restimulated overnight with 50 µg/ml of CII (T-Cell Proliferation; Chondrex). The next day, cells were stimulated with 25 ng/ml PMA and 1 µg/ml ionomycin for an additional 3 h. Cells were harvested, washed, stained and analyzed as previously described (48, 60, 61).

To measure inflammatory cells in the arthritic joints, isolated limb joints were digested with 2 mg/ml collagenase (*Clostridium histolyticum*, Type IV; Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C, and cell suspensions passed through a 70 µm cell strainer similar to that previously described (40, 62). Leukocytes were stained and analyzed by forward and side-scatter plots for $Ly-6G^+$ $Ly-6C^+$ $CD11b^+$ neutrophils.

Statistics

Mann-Whitney U-test was applied to statistically analyze clinical scores. The difference in arthritis incidence between experimental groups was checked with Fisher's exact probability test. One-way ANOVA was performed to analyze ELISA and flow cytometry results. Data were considered statistically significant, if p-value was < 0.05.

RESULTS AND DISCUSSION

RA is a chronic, systemic autoimmune disorder affecting millions of patients in the US. Treatment of this progressive, degenerative disease demands constant use of anti-inflammatory drugs and often immunosuppressive treatments that increase susceptibility to infections and neoplasia (4, 15, 16). Instead, intervention strategies that focus on redirecting or reeducating T cell responses to produce tolerance instead of inflammation have the potential of being a superior treatment for RA.

To address the void for such tolerance induction, we queried whether a probiotic LAB engineered to express the potent anti-inflammatory cytokine, IL-35 (**Figure 1A**), would diminish arthritis. Expression of IL-35 by LL-IL35 was detected by Western blot analysis using a rabbit polyclonal serum against an MBP-IL-35 fusion protein [produced in-house; (61)]. To test the therapeutic properties of LL-IL35, mice were challenged on day 0 with CII to induce CIA. Given its similarity, CIA is often exploited as an investigative tool to test novel strategies and therapeutics to prevent and treat RA. These mice were randomly divided into three groups for oral treatment: LL-IL35 (pBzMM150), LL vector (pMSP3535H3), or sterile PBS. Two treatment paradigms were tested: beginning intervention on day 14 resembling *Salmonella*-CFA/I treatment (60) with two additional doses on days 21 and 28 (**Figure 1B**) or beginning intervention at disease onset on day 18, followed by a second dose on day 25 (**Figure 1C**). Clinical scores were performed in double blind, and followed until day 39 post-induction. We generally do not see changes in disease severity beyond 39 days post-CII challenge.

Using the three-dose regimen, 50% of the LL-IL35-treated mice showed no symptoms and the remaining 50% developed minor symptoms as opposed to PBS- or LL vector-treated mice, who all developed severe arthritis by day 24 post-CII challenge. Notably, the severity of disease symptoms was significantly less ($p < 0.001$) in the LL-IL35-treated mice exhibiting an average clinical score of 1 in contrast to PBS- or LL vector-treated mice eventually achieving clinical scores of ∼9 (**Figure 1B**). To test if LL-IL35 is effective in arresting the disease after disease onset, additional groups of CIA mice were treated using a two-dose regimen on days 18 and 25. Under this treatment, 40% of the LL-IL35-treated mice developed CIA vs. 100% of those treated with PBS or LL vector (**Figure 1C**). Compared to the three-dose regimen, the disease severity was greater for the LL-IL35-treated CIA mice subjected to the two-dose regimen, although significantly less ($p < 0.05$) when compared to similarly treated PBS- or LL vector-dosed mice. These data show that LL-IL35 can effectively reduce the symptoms of arthritis and the incidence of disease via its immunosuppressive capacity. Moreover, these results show that fewer doses of IL-35 delivered by LL are needed to curtail arthritis when compared to treatment with soluble protein (47, 48, 51, 52).

Analysis of knee joints was performed to determine the extent of neutrophil infiltration. In agreement with these clinical findings, the LL-IL35-treated mice had markedly reduced $Ly-6G^+$ $CD11b^+$ cells (neutrophils) infiltrating the joints (**Figure 2A**) representing a 7- and 5-fold reduction compared to
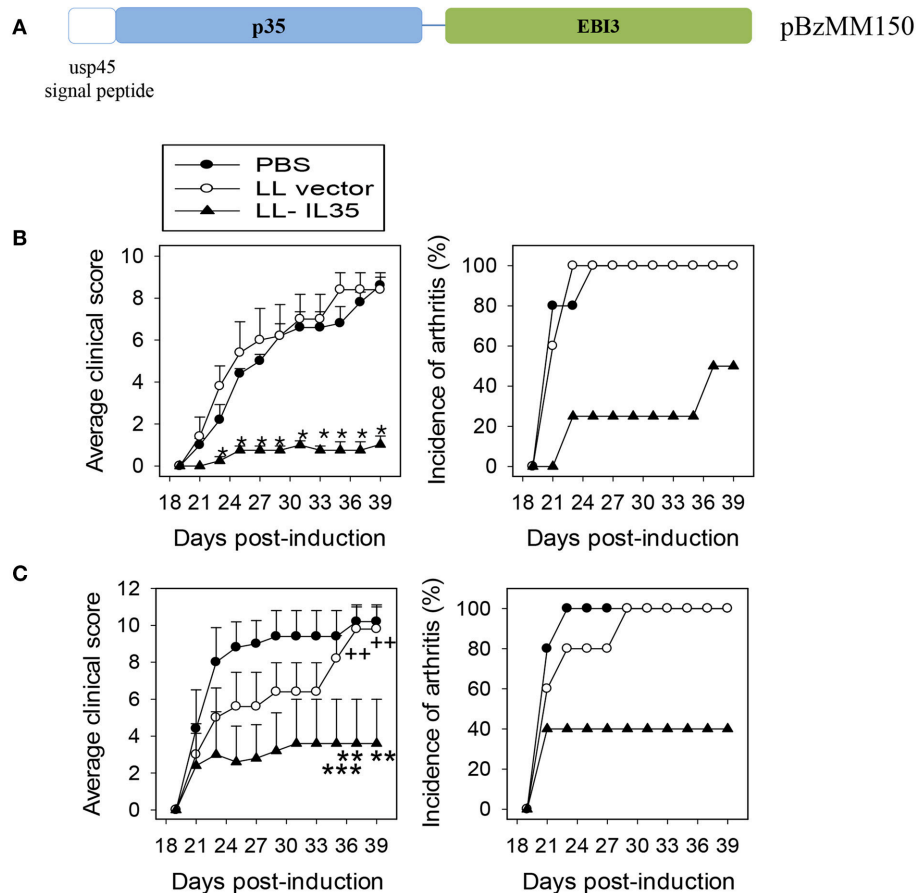
FIGURE 1 | IL-35 inhibits CIA progression following treatment with LL-IL35. **(A)** Schematic map of the synthetic DNA used to construct pBzMM150 for murine IL-35 expression of IL-35 in *L. lactis*. The synthetic insert encodes in order: the usp45 secretion peptide genetically fused in-frame to the p35 subunit, a short flexible linker, fused to the EBI3. The synthetic DNA also features an optimal Shine-Dalgarno (SD) sequence at the optimal distance from the ATG initiation codon, in addition to the SD sequence present in the vector. The nisin-inducible promoter and a transcription terminator are borne on the expression vector pMSP3535H3. **(B,C)** CIA was induced in groups of C57BL/6 males with chick CII emulsified in complete Freund's adjuvant. Two regimens were tested: orally treated with 5×10^8 CFUs of LL vector or LL-IL35 or sterile PBS on **(B)** 14, 21, and 28 days or **(C)** 18 and 25 days post-induction. Average clinical score per treatment group (left panels) represents severity of the disease, and incidence of arthritis depicts percent mice with affected joints in each treatment group (right panels). The sum of 10 mice/group is shown: **(B)** $^*p < 0.001$ vs. PBS-dosed or LL vector-treated mice, and **(C)** $^{**}p < 0.02$, $^{***}p < 0.05$ vs. PBS-dosed mice and $^{++}p < 0.02$ vs. LL vector-treated mice .

PBS-dosed or LL vector-treated groups, respectively (**Figure 2B**). Hence, IL-35 can reduce inflammation of the joints in CIA-challenged mice.

To investigate the possible mechanism of protection conferred by LL-IL35, CD4$^+$ T cells purified from draining LNs were anti-CD3 + anti-CD28-restimulated and analyzed for cytokine production. These CD4$^+$ T cells were obtained from mice dosed three times with PBS, LL vector, or LL-IL35 as described in **Figure 1B**. IFN-γ levels remained elevated between PBS-dosed and LL vector-treated mice, in contrast to LL-IL35-treated mice showing 3- to 3.7-fold reduction ($p < 0.001$; **Figure 3A**). Concomitantly, IL-17 levels were significantly less ($p < 0.001$) by 3.7- and 8.5-fold for LL vector and LL-IL35-treated groups, respectively, relative to PBS-dosed mice (**Figure 3B**). Moreover, treatment with LL-IL35 significantly reduced IL-17 by 2.3-fold compared to LL vector-treated mice ($p < 0.01$). Minimal stimulation of IL-10 was detected in the restimulated CD4$^+$

T cells from the PBS-dosed mice (**Figure 3C**). In contrast, CD4$^+$ T cells from LL vector- and LL-IL35-treated groups showed significantly increased IL-10 production ($p < 0.05$) by 1.8- and 2.5-fold, respectively. The difference between the LL vector- and LL-IL35-treated groups was significant ($p < 0.05$; **Figure 3C**) as well. The stimulation of IL-10 by LL vectors has been reported by others (56, 57). However, IL-10 induced by the LL vector-treated group was insufficient to suppress disease progression (**Figure 1**) and IL-17 production (**Figure 3B**). IL-10's importance for suppressing CIA was previously demonstrated in IL-10$^{-/-}$ mice with CIA being refractory to IL-35 treatment (48), supporting the notion here of IL-10's relevance to CIA mice treated with LL-IL35. IL-35 has also been shown to stimulate IL-10 production (40, 47–49).
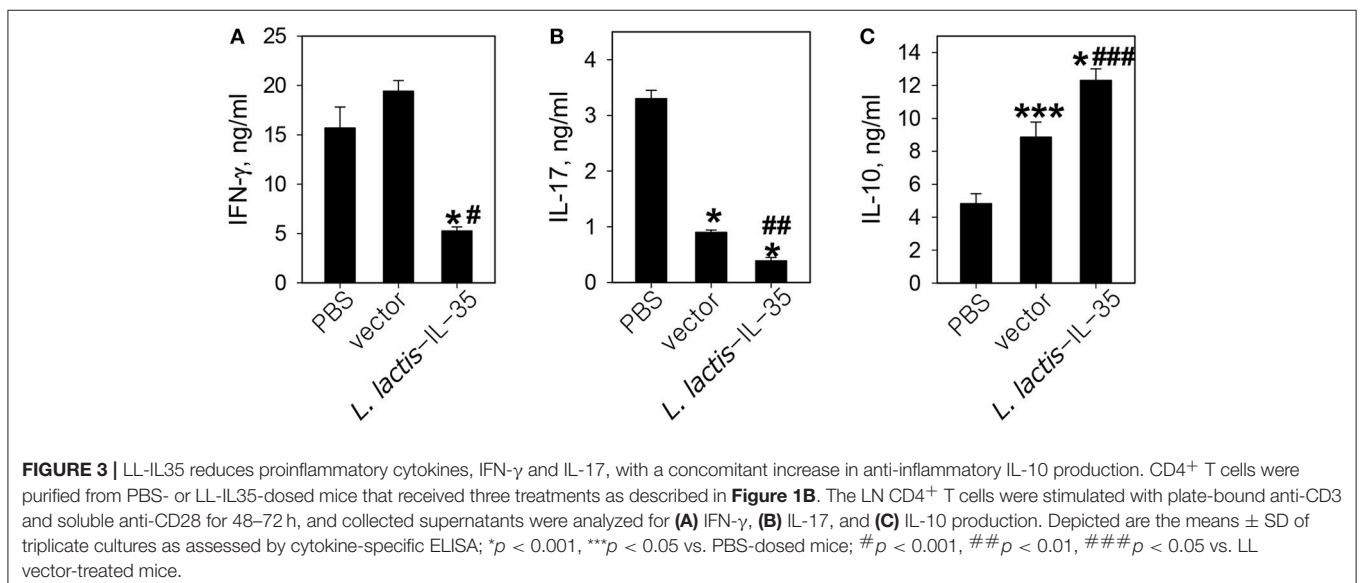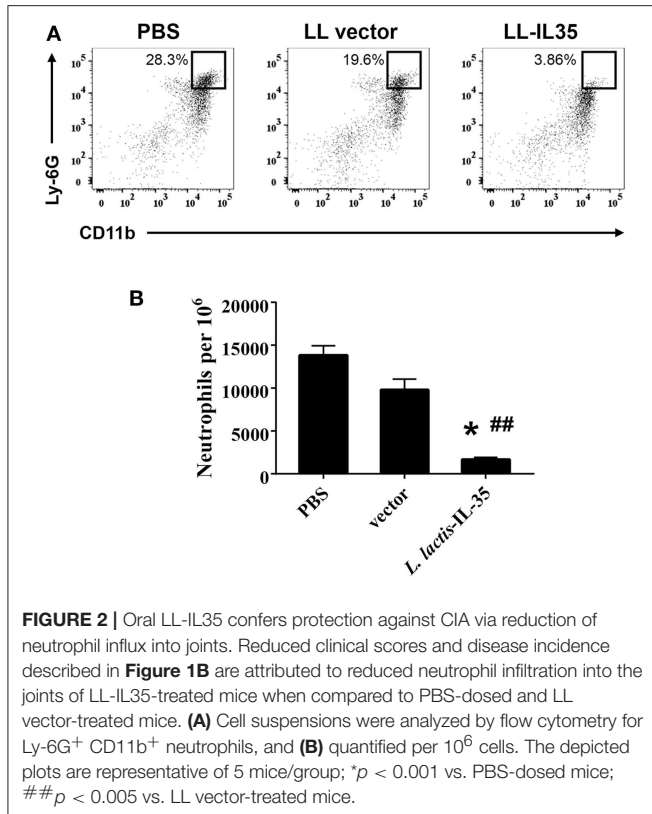
We have found that CD39$^+$ CD4$^+$ T cells are the primary Tregs responsible for resolving CIA (48, 60, 61). CD39 is an ectonucleoside triphosphate diphosphohydrolase-1which

hydrolyzes ATP into AMP, thus quenching inflammatory signaling by extracellular ATP (58, 63). We also showed that $CD25^+$ Tregs remained a subset of $CD39^+$ $CD4^+$ T cells, and that CD39 encompassed all of the Treg subsets (60). In fact, $CD39^+$ Tregs were protective against CIA (40, 60). These Tregs are composed of two subsets, $Foxp3^+$ and $Foxp3^-$, and are

interchangeable (60). Analysis of induction of $CD39^+$ Tregs by the LL vector revealed no increase in the percentage of these Tregs in CIA mice (40), and CIA had only a modest impact upon their induction (40, 61).

To examine the types of Tregs induced by LL-IL35 treatment, whole splenic and draining LN lymphocytes were cultured overnight with CII, and then pulsed with PMA + ionomycin to ascertain the type of Tregs induced in PBS-dosed and LL-IL35-treated mice. Lymphocytes were then stained for CD39, Foxp3, and CCR6 to identify the Treg subsets. Since CCR6 has been shown to be expressed by Tregs (53–55), we queried whether such Tregs may be induced as a consequence of IL-35 treatment. $CD39^+$ $CD4^+$ T cells were evaluated for expression of Foxp3 and CCR6 (**Figures 4A,B**). Upon examination of splenic Tregs derived from PBS-dosed mice compared to those present in LL-IL35-treated mice, a modest increase ($p < 0.05$) in the frequency, but not the total number of $CCR6^+$ $Foxp3^+$ $CD39^+$ $CD4^+$ T cells, was observed (**Figures 4C,G**). A modest difference ($p \leq 0.01$) was also observed in the frequency and total number of splenic $CCR6^+$ $Foxp3^-$ $CD39^+$ $CD4^+$ T cells when compared to the PBS-dosed mice (**Figures 4E,I**). However, when similar analysis was performed for Tregs obtained from the draining LNs, a 2.2-fold increase in the frequency ($p < 0.001$) of $CCR6^+$ $Foxp3^+$ $CD39^+$ $CD4^+$ T cells was stimulated by LL-IL35 treatment compared to those present in PBS-dosed CIA mice (**Figure 4D**). The total number of these LN Tregs was also significantly ($p \leq 0.01$) increased by 2.7-fold (**Figure 4H**). Subsequent analysis was performed on LN $CCR6^+$ $Foxp3^-$ $CD39^+$ $CD4^+$ T cells, and both the frequency and total number increased significantly by 2.9- ($p \leq 0.01$) and 5.1-fold ($p < 0.001$), respectively (**Figures 4F,J**). These studies demonstrate that indeed $CCR6^+$ Tregs are induced by IL-35 treatment of CIA mice.

Additional analyses were performed on both $CCR6^-$ $Foxp3^+$ and $CCR6^-$ $Foxp3^-$ $CD39^+$ $CD4^+$ T cells (**Figures 4K–R**). Examination of the splenic $CCR6^-$ $Foxp3^+$ $CD39^+$ $CD4^+$ T

FIGURE 2 | Oral LL-IL35 confers protection against CIA via reduction of neutrophil influx into joints. Reduced clinical scores and disease incidence described in **Figure 1B** are attributed to reduced neutrophil infiltration into the joints of LL-IL35-treated mice when compared to PBS-dosed and LL vector-treated mice. **(A)** Cell suspensions were analyzed by flow cytometry for $Ly-6G^+$ $CD11b^+$ neutrophils, and **(B)** quantified per 10^6 cells. The depicted plots are representative of 5 mice/group; $*p < 0.001$ vs. PBS-dosed mice; $\#\#p < 0.005$ vs. LL vector-treated mice.

FIGURE 3 | LL-IL35 reduces proinflammatory cytokines, IFN-γ and IL-17, with a concomitant increase in anti-inflammatory IL-10 production. $CD4^+$ T cells were purified from PBS- or LL-IL35-dosed mice that received three treatments as described in **Figure 1B**. The LN $CD4^+$ T cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 for 48–72 h, and collected supernatants were analyzed for **(A)** IFN-γ, **(B)** IL-17, and **(C)** IL-10 production. Depicted are the means ± SD of triplicate cultures as assessed by cytokine-specific ELISA; $*p < 0.001$, $***p < 0.05$ vs. PBS-dosed mice; $\#p < 0.001$, $\#\#p < 0.01$, $\#\#\#p < 0.05$ vs. LL vector-treated mice.
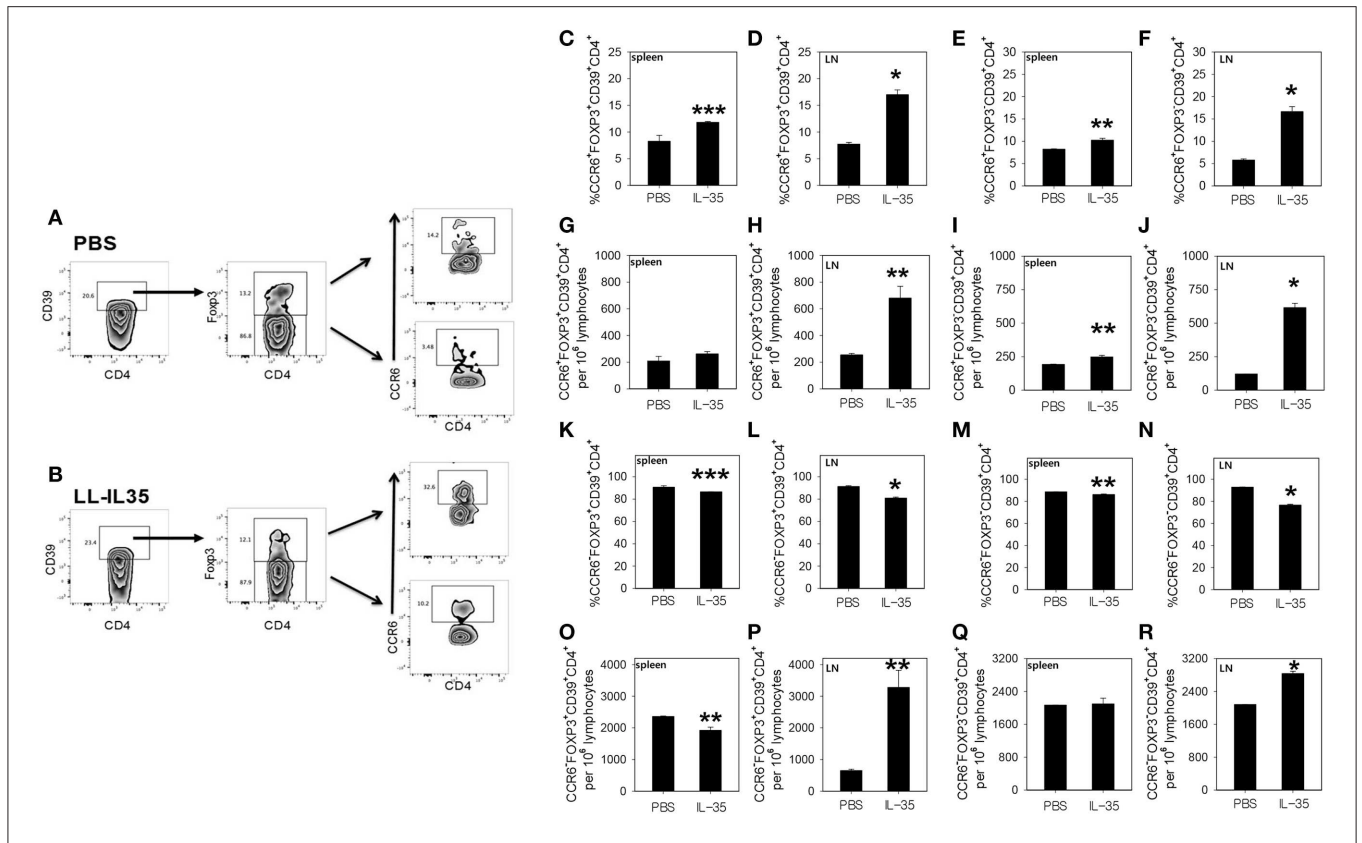
FIGURE 4 | LL-IL35 induces CCR6$^+$ and CCR6$^-$ CD39$^+$ CD4$^+$ T cells in CIA mice. At the termination of the study, whole splenic and LN lymphocytes were restimulated with 50 µg/ml CII overnight, and then subjected to a short-term of PMA + ionomycin. LN CD39$^+$ CD4$^+$ T cells from **(A)** PBS-dosed and **(B)** LL-IL35-treated mice were gated on Foxp3$^+$ and Foxp3$^-$ cells, and analyzed for **(C–R)** for CCR6 expression by **(C,E,G,I,K,M,O,Q)** splenic and **(D,F,H,J,L,N,P,R)** LN lymphocytes. **(C,D)** Frequency of CCR6$^+$ Foxp3$^+$ and **(E,F)** CCR6$^+$ Foxp3$^-$ and absolute **(G,H)** CCR6$^+$ Foxp3$^+$ and **(I,J)** CCR6$^+$ Foxp3$^-$ T cells are shown. **(K,L)** Frequency of CCR6$^-$ Foxp3$^+$ and **(M,N)** CCR6$^-$ Foxp3$^-$ and absolute **(O,P)** CCR6$^-$ Foxp3$^+$ and **(Q,R)** CCR6$^-$ Foxp3$^-$ T cells are also shown. Depicted are the means ± SEM of 5 mice/group; *$p < 0.001$, **$p \leq 0.010$, and ***$p < 0.05$ compared with PBS-dosed mice.
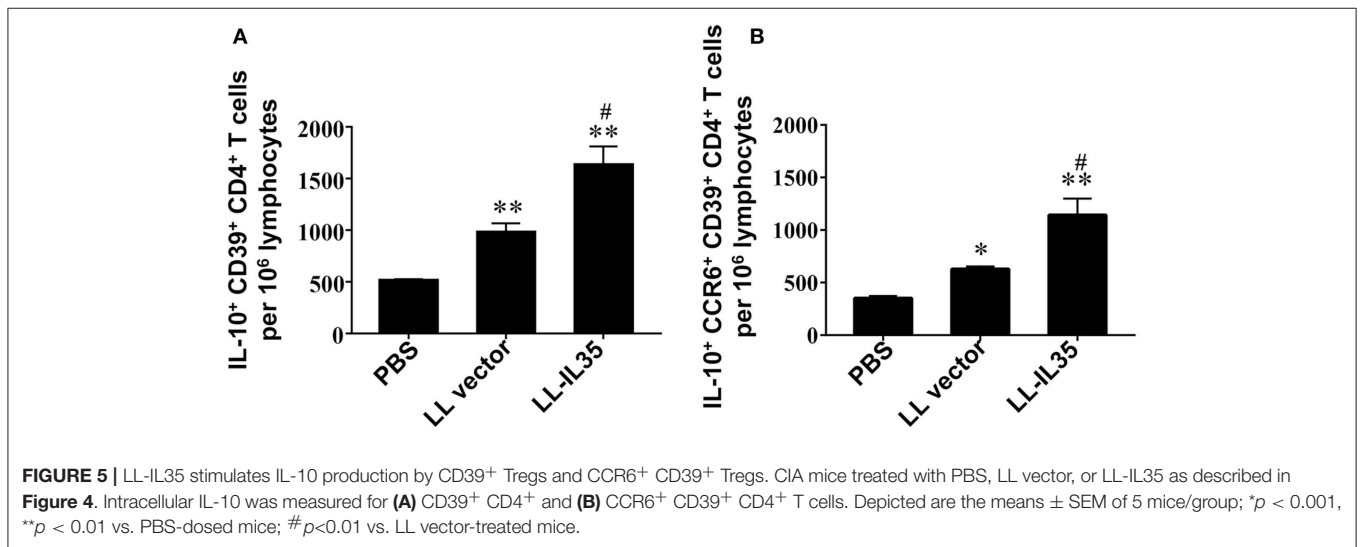

FIGURE 5 | LL-IL35 stimulates IL-10 production by CD39$^+$ Tregs and CCR6$^+$ CD39$^+$ Tregs. CIA mice treated with PBS, LL vector, or LL-IL35 as described in **Figure 4**. Intracellular IL-10 was measured for **(A)** CD39$^+$ CD4$^+$ and **(B)** CCR6$^+$ CD39$^+$ CD4$^+$ T cells. Depicted are the means ± SEM of 5 mice/group; *$p < 0.001$, **$p < 0.01$ vs. PBS-dosed mice; #$p<0.01$ vs. LL vector-treated mice.

cells revealed that both the frequency and total number were modestly and significantly ($p < 0.05$) reduced for the LL-IL35-treated mice (**Figures 4K,O**). The frequency of splenic CCR6$^-$

Foxp3$^-$ CD39$^+$ CD4$^+$ T cells was slightly and significantly ($p \leq 0.01$) reduced (**Figure 4M**), but the total number of these CD39$^+$ CD4$^+$ T cells showed no difference between PBS-dosed

and LL-IL35-treated CIA mice (**Figure 4Q**). Similar analysis was also performed for the LN CCR6$^-$ Foxp3$^+$ and CCR6$^-$ Foxp3$^-$ CD39$^+$ CD4$^+$ T cells from the same treated CIA mice. While a slight reduction in the frequency of LN CCR6$^-$ Foxp3$^+$ CD39$^+$ CD4$^+$ T cells was observed for LL-IL35-treated mice (**Figure 4L**), the total number of CCR6$^-$ Tregs was significantly ($p \leq 0.01$) elevated by 5-fold (**Figure 4P**). Examination of the frequency of LN CCR6$^-$ Foxp3$^-$ CD39$^+$ CD4$^+$ T cells also showed a modest, but significant ($p < 0.001$) reduction in LL-IL35-treated mice relative to PBS-dosed mice (**Figure 4N**), but the total number of these LN T cells was significantly ($p < 0.001$) enhanced by 36% (**Figure 4R**). Hence, these analyses demonstrate that IL-35 treatment stimulates diverse subsets of Tregs including both CCR6$^+$ and CCR6$^-$ Tregs. Future studies will need to consider the longevity of these subsets for protection against CIA.

Inquiring into the activity of these LN CD39$^+$ Tregs, analysis for IL-10 production was performed (**Figure 5**). Intracellular IL-10 measurements were conducted first for all CD39$^+$ CD4$^+$ T cells (both Foxp3$^+$ and Foxp3$^-$). The LL-IL35-treated mice showed 3.2- and 1.7-fold more IL-10-producing cells ($p \leq 0.01$) than PBS-dosed and LL vector-treated CIA mice, respectively (**Figure 5A**). LL vector-treated mice showed 1.9-fold increase in the number of IL-10-producing CD39$^+$ CD4$^+$ T cells compared to PBS-dosed mice ($p \leq 0.01$; **Figure 5A**). Examination of IL-10$^+$ CCR6$^+$ CD39$^+$ CD4$^+$ T cells (both Foxp3$^+$ and Foxp3$^-$) revealed that two-thirds of the total IL-10-producing cells induced by LL-IL35 treatment of CIA mice were derived from the CCR6$^+$ subset (**Figure 5B**). The CCR6$^+$ CD39$^+$ CD4$^+$ T cells induced with LL-IL35 resulted in significant 3.2- and 1.8-fold increase in IL-10-producing cells than those present in PBS-dosed ($p \leq 0.01$) and LL vector-treated CIA mice ($p < 0.01$), respectively. LL vector-treated mice showed 1.8-fold increase in the number of IL-10-producing CCR6$^+$ CD39$^+$ CD4$^+$ T cells compared to PBS-dosed mice ($p \leq 0.01$; **Figure 5B**). These findings suggest that indeed both Foxp3$^+$ and Foxp3$^-$ CCR6$^+$ CD39$^+$ Tregs are the predominant source of IL-10, thus contributing to the amelioration of CIA subsequent LL-IL35 treatment. Such finding may mimic what is evident with human peripheral blood CCR6$^+$ CD39$^+$ Tregs (64) and CCR6$^+$ Tregs found in patients with glomerulonephritis (65).

The data presented demonstrate the potency of IL-35 as an anti-inflammatory therapeutic. Moreover, this investigation further supports the multifaceted benefits of adapting recombinant *L. lactis* as a vector to deliver therapeutic doses of IL-35. In fact, previous studies by us (48) or others, using IL-35 to treat type 1 diabetes model (51), IBD (52), or psoriasis (52), required daily treatments with recombinant protein to control disease. In contrast, only two or three oral doses of LL-IL35 were sufficient to prevent the onset or stop CIA progression. Oral dosing has the substantial advantage of being less invasive circumventing the need for injections. LL-derived IL-35 eliminates the labor-intensive efforts needed to produce and purify the recombinant protein, dramatically reducing the cost of manufacturing this therapeutic. Moreover, IL-35 is a dimeric protein which adds to the difficulty and cost to generate. The *L. lactis* used for this study is a lab-adapted recombinant strain, originally derived from an industrial dairy strain capable of fermenting milk into a product that has the same textural and olfactory properties of yogurt (40). We previously have demonstrated that the curative properties of our recombinant *L. lactis* are maintained when grown on a synthetic medium or used to ferment milk into a yogurt-like product (40). These attributes make *L. lactis* an ideal tolerogen delivery platform for the treatment of autoimmune diseases.

AUTHOR CONTRIBUTIONS

MM, IK, and DP: conceptualization. MM, IK, CH, and DP: formal analysis, investigation, methodology, validation, visualization, and writing. DP: funding acquisition. All authors approved the final version to be published and agreed to the content and all aspects of the work ensuring the accuracy or integrity of the work are appropriately investigated and presented.

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