Immunomodulation by *Bifidobacterium infantis* 35624 in the Murine Lamina Propria Requires Retinoic Acid-Dependent and Independent Mechanisms

Patrycja Konieczna¹, Ruth Ferstl¹, Mario Ziegler¹, Remo Frei¹,², Dirk Nehrbass³, Roger P. Lauener²,⁴, Cezmi A. Akdis¹,², Liam O’Mahony¹*

¹Swiss Institute of Allergy and Asthma Research (SIAF), University of Zurich, Davos, Switzerland, ²Christine Kühne-Center for Allergy Research and Education (CK-CARE), Davos, Switzerland, ³AO Research Institute Davos (ARI), Davos, Switzerland, ⁴Hochgebirgsklinik Davos-Wolfgang, Davos, Switzerland

**Abstract**

Appropriate dendritic cell processing of the microbiota promotes intestinal homeostasis and protects against aberrant inflammatory responses. Mucosal CD103⁺ dendritic cells are able to produce retinoic acid from retinal, however their role in vivo and how they are influenced by specific microbial species has been poorly described. *Bifidobacterium infantis* 35624 (*B. infantis*) feeding to mice resulted in increased numbers of CD103⁺ retinaldehyde dehydrogenase (RALDH)⁺ dendritic cells within the lamina propria (LP). Foxp3⁺ lymphocytes were also increased in the LP, while T₃₁ and T₃₁₇ subsets were decreased. 3,7-dimethyl-2,6-octadienal (citral) treatment of mice blocked the increase in CD103⁺ RALDH⁺ dendritic cells and the decrease in T₃₁ and T₃₁₇ lymphocytes, but not the increase in Foxp3⁺ lymphocytes. *B. infantis* reduced the severity of DSS-induced colitis, associated with decreased T₃₁ and T₃₁₇ within the LP. Citral treatment confirmed that these effects were RALDH mediated. RALDH⁺ dendritic cells decreased within the LP of control inflamed animals, while RALDH⁺ dendritic cells were maintained in the LP of *B. infantis*-fed mice. Thus, CD103⁺ RALDH⁺ LP dendritic cells are important cellular targets for microbiota-associated effects on mucosal immunoregulation.

**Citation:** Konieczna P, Ferstl R, Ziegler M, Frei R, Nehrbass D, et al. (2013) Immunomodulation by *Bifidobacterium infantis* 35624 in the Murine Lamina Propria Requires Retinoic Acid-Dependent and Independent Mechanisms. PLoS ONE 8(5): e62617. doi:10.1371/journal.pone.0062617

**Editor:** Stefan Bereswill, Charité-University Medicine Berlin, Germany

**Received December 19, 2012; Accepted March 23, 2013; Published May 21, 2013**

**Copyright:** © 2013 Konieczna et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** The authors are supported by Swiss National Foundation grants (project numbers 32030-132899 and 310030-127356), Christine Kühne Center for Allergy Research and Education (CK-CARE) and European Union (EU) Marie Curie grants. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have the following conflicts. Liam O’Mahony is a consultant to Alimentary Health Ltd. Cezmi A. Akdis has received research support from Novartis and Stallergenes and consulted for Actellion, Aventis and Allergopharma. Patrycja Konieczna, Mario Ziegler, Dirk Nehrbass, Remo Frei, Ruth Ferstl and Roger P. Lauener have no conflict of interest. The authors do not have any additional financial or non-financial disclosures, which have not already been described online. This does not alter their adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: liam.omahony@siaf.uzh.ch

**Introduction**

The mammalian gastrointestinal microbiota is required for optimal host development and ongoing immune homeostasis [1–3]. The microbiota aids in the digestion of foods, competes with pathogens, degrades mucus and promotes the differentiation of epithelial cells and mucosa-associated lymphoid tissue. In addition, the composition and metabolic activity of the microbiota has profound effects on proinflammatory activity and the induction of immune tolerance by influencing a broad range of mucosal cell types including epithelial cells, dendritic cells, iNKT cells and T lymphocyte subset activity [4–6].

Gastrointestinal immune homeostasis is dependent on a number of local conditioning factors that reduce pathological proinflammatory responses to non-pathogenic microbes. For example, epithelial-derived cytokines such as TSLP and IL-25 limit dendritic-cell secretion of IL-12 and IL-23, while promoting IL-10 secretion [7]. In addition, certain dendritic cell subsets within the mucosa can metabolize vitamin A into retinoic acid, such as the CD103⁺ dendritic cell subset [8,9]. Retinoic acid is synthesized from stored or dietary retinol by the oxidation of retinol to retinal, followed by oxidation of retinal to retinoic acid. The final step is catalyzed by aldehyde dehydrogenase family 1, subfamily A1 (Aldh1a1) and ALDH1 subfamily A2 (Aldh1a2), also called RALDH enzymes. 3,7-dimethyl-2,6-octadienal (citral) blocks RALDH enzymatic activity. Dendritic cell-derived retinoic acid has dramatic effects on dendritic cell activity and lymphocyte subset plasticity. Retinoic acid can have seemingly conflicting effects on lymphocyte polarization, such as promoting TH17 cells or Treg cells [10]. The promotion of T₃₁ and T₃₁₇ phenotypes may be related to the local concentration of retinoic acid, the dendritic cell subset secreting retinoic acid, the local level of pro-inflammatory mediators and TGF-β, comitant toll-like receptor activation or induction of specific microRNA [11–14]. So far, the role of specific microbial species in influencing retinoic acid metabolism and CD103⁺RALDH⁺ dendritic cells in vivo has been poorly understood.

*Bifidobacterium longum* subsp. *infantis* 35624 (*B. infantis*) was originally isolated from resected human healthy gastrointestinal tissue and human clinical studies have demonstrated its efficacy in Irritable Bowel Syndrome patients [15,16]. In addition, murine studies have demonstrated that this microbe protects against inflammatory disorders across a range of inflammatory conditions including colitis, pathogen infection, arthritis and respiratory
inflammation [17–20]. Previously, in vitro studies with human dendritic cells suggested that promotion of retinoic acid metabolism by *B. infantis* was a key regulatory feature of this bacterium [21]. In this report, we demonstrate that *B. infantis* feeding to mice results in increased CD103+RALDH+ dendritic cells within the mucosa, which are responsible for the suppression of Th1 and Th17 lymphocytes and amelioration of dextran sulfate sodium (DSS)-induced colitis.

**Methods**

**Bacteria and animal models**

Wild-type C57BL/6 mice were obtained from Charles River and maintained under specific pathogen free conditions. Mice were housed at the AO Research Institute, Davos, Switzerland, in individually ventilated cages for the duration of the study, and all experimental procedures were carried out in accordance with Swiss law. Experimental protocols were approved by the Ethics Committee of the “Amt für Lebensmittelsicherheit und Tiergesundheit Graubünden”, application number 2011-13. In the first experiment, three groups of mice were utilized (n = 8 per group). Group 1 did receive any bacterial supplementation, while groups 2 and 3 were fed *B. infantis* for 7 days. Each day hypophilized bacteria were resuspended in sterile water to final concentration of 6×10⁶ colony forming units (cfu)/ml. For group 3, 2 mg of citral (Sigma, St. Louis, USA) was dissolved in 10% DMSO (Sigma) and was injected i.p. daily in order to suppress retinoic acid metabolism.

In the dextran sodium sulfate (DSS) colitis model, five groups of wild-type C57BL/6 mice (n = 8 per group) were utilized. Group 1 was the negative control group, which did not receive *B. infantis* and were not administered DSS. Group 2 was the positive control group as these mice received DSS but not *B. infantis*. Groups 3 and 4 were both administered DSS and *B. infantis*, while group 4 was also injected i.p. with citral (as described above). Group 5 received DSS and citral. Mice were fed *B. infantis* for 7 days before colitis induction. Mice received DSS (TdB Consultancy AB, Uppsala) in water (2.5%) for 6 days followed by 2 days without DSS. During this period bacteria were administrated daily by gavage (1×10⁹ cfu/mouse). All mice were euthanized on the final day of the study using cervical dislocation, which was performed by an experienced investigator.

**Cell isolation**

Single cell suspensions from mesenteric lymph nodes (MLN) and Peyer’s patches (PP) were isolated using C tubes and GentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer instructions. LP cells were isolated from the upper part of small intestine (SI). A 5 cm long piece of SI was washed out with cold calcium and magnesium free PBS (CMF-PBS) containing 1 mmol dithiothreitol (DTT) and cold CMF-PBS containing 12 mmol EDTA. The SI was cut into pieces and vortexed in CMF-PBS containing 0.3 mmol EDTA. After centrifugation (300 g/5 minutes) tissue was digested for 45 minutes at 37°C in RPMI containing 25 kU/l collagenase IV (Sigma), 150 mg/l DNase I (Roche, Rotkreuz, Switzerland) and 5% fetal calf serum (FCS, Sigma). Cell suspensions were filtered through 70 µm cell strainers, centrifuged (700 g/8 minutes) and washed with CMF-PBS containing 5% FCS, 5 mg/l DNase I, 5 mmol/l EDTA. Finally pellets were resuspended in cRPMI (Invitrogen, LuBioScience, Luzern, Switzerland).

**Flow cytometry and cell imaging**

Anti-mouse CD11b, CD11c, MHCIi, CD3, CD19 and CD103 antibodies (Biolegend, Lucerna-Chem, Luzerna, Switzerland) were used for characterization of dendritic cell phenotypes. RALDH activity was measured with the ALDEFLOUR kit (Aldagen, Durham, USA) according to manufacturer instructions. Anti-mouse CD3, CD4, CD25, LPAM-1 (integrin α4β7) and CCR9 antibodies (Biolegend) and anti-mouse Foxp3, IL-10, IL-4, IL-17A, IFNγ antibodies (eBioscience San Diego, CA, USA) were used to characterize lymphocyte phenotypes. Cells for intracellular cytokine staining were pre-stimulated for 4 hours with PMA (50 ng/ml, Sigma) and ionomycin (500 ng/ml, Sigma) in the presence of Brefeldin A (1 µg/ml, eBioscience). Flow cytometric analysis was performed using a 10 colour Galios flow cytometer (Beckman Coulter, Brea, USA). Kaluza (Beckman Coulter) was used for data analysis.

MLN and LP cells were incubated in vitro for 1 hour with CFSE labeled *B. infantis*. Cells were stained with anti-mouse CD11c and CD103. Bacteria binding was visualized using multispectral imaging flow cytometer Image Stream X (Amnis Corporation, Seattle, USA) and images were analyzed using IDEAS software (Amnis Corporation).

**Histology**

Colon sections were fixed in 4% paraformaldehyde for 12 hours and stored in PBS until paraffin embedding. Following embedding, 3 µm thick sections were stained with Gill’s hematoxylin and eosin (Sigma). Mounting was done with Eukitt® quick-hardening mounting medium (Sigma). Tissue samples were analyzed by a pathologist in a blinded manner. The histology score included assessment of crypts dilatation, inflammatory cells infiltration in LP, inflammatory cells infiltration in submucosa, necrosis of epithelium and submucosal edema. Each parameter was scored from 0 to 5 resulting in a maximum score of 25.

**Myeloperoxidase (MPO) activity test**

Pieces of colon were homogenized in 50 mM potassium phosphate buffer, pH 6.0, containing 0.3% hexadecyltrimethyl-ammonium bromide (HTAB, Sigma). Tissue was sonicated on ice for 15 seconds followed by 3 freeze-thaw cycles. Samples were centrifuged and 20 µl of the supernatant was mixed with 200 µl freshly prepared 50 mM potassium phosphate buffer, pH 6.0, containing 0.3% HTAB, O-dianisidine dihydrochloride (0.167 mg/ml, Sigma) and 0.5% hydrogen peroxide (Sigma). Change in absorbance was measured at 450 nm over 4 min. by an ELISA plate reader.

**Cytokine assay**

1×10⁶ SI-LP cells were cultured in 1 ml cRPMI containing amphotericin B (6.25 µg/ml, Sigma) and gentamycin (12.5 µg/ml, Sigma) and supernatants were collected after 24 hours. Cytokine secretion was examined by Bio-Plex multiplex suspension array (Bio-Rad Laboratories, Hercules, USA).

**Statistics**

Unpaired student t-tests were used to analyse data with a normal distribution, while the non-parametric Mann-Whitney test was used to analyze the non-parametric data. All data analysis was carried out using GraphPad Prism software. A p value of <0.05 was used as the cutoff for statistical significance.
Results

*B. infantis* is sampled by Peyer’s patch and lamina propria dendritic cells

Dendritic cells within the LP and Peyer’s patches (PP) have been previously described to sample bacteria from the gastrointestinal lumen. In order to determine whether *B. infantis* was sampled by dendritic cells from either site, CFSE-labelled bacteria were gavaged to mice and single cell suspensions were generated from ileal LP and PP after 2 hours. Within the PP, CD11c^+^MHCI^+^ dendritic cells were identified, which had become CFSE positive 2 hours after feeding (Figure 1). CD11c^+^MHCI^+^ dendritic cells within the LP also became CFSE positive at 2 hours and with higher frequency (Figure 1). The presence of CFSE-labelled bacteria attached to, or internalized by, PP dendritic cells at 2 hours was confirmed using multispectral flow cytometry imaging (Figure 1).

*B. infantis* induces CD103^+^ retinoic acid secreting dendritic cells

We examined CD103 expression and retinoic acid secretion by dendritic cells in mesenteric lymph nodes (MLN) and ileal LP following *B. infantis* feeding for 7 days. *B. infantis* feeding was associated with a significant increase in the percentage of dendritic cells that were CD103^+^ and metabolizing retinoic acid in the LP, while the increase in retinoic acid metabolizing CD103^+^ dendritic cells in the MLN approached statistical significance (Figure 2). Citral treatment, which blocks retinoic acid metabolism, reduced the *B. infantis*-induced increase in CD103^+^ and retinoic acid metabolizing dendritic cells within the LP. In order to determine if the increase in retinoic acid metabolism was a direct effect of *B. infantis* binding to CD103^+^ dendritic cells, *in vitro* co-incubation with mucosal dendritic cells demonstrated that CD103^+^ dendritic cells were able to bind *B. infantis* at a high frequency, while CD103^-^ dendritic cells bound *B. infantis* at a low frequency (Figure 2 and Figure S1). Moreover, CD103^+^ dendritic cells gene expression of the retinoic acid metabolizing enzymes ALDH1a1 and ALDH1a2 were significantly upregulated following co-incubation with *B. infantis* (Figure 2). In addition, CD103^+^ and CD103^-^ dendritic cells were isolated from the mucosa by flow cytometric sorting and were co-incubated with *B. infantis*. Gene expression for ALDH1a1 and ALDH1a2 was significantly increased following *B. infantis* co-incubation only in CD103^+^ dendritic cells, but not CD103^-^ dendritic cells (Figure 2).

*B. infantis* suppression of T~17~ cells is retinoic acid-dependent

Ileal LP single cell suspensions were examined for IL-17, IFN-gamma and IL-4 positive lymphocytes. The percentage of IL-17^+^ lymphocytes within the LP was significantly reduced following *B. infantis* feeding for 7 days, while the reduction in IFN-gamma^+^ lymphocytes approached statistical significance (Figure 3). Citral treatment reversed the *B. infantis* suppression of IL-17^+^ and IFN-gamma^+^ lymphocytes within the LP. Neither *B. infantis* feeding nor citral treatment altered the percentage of IL-4^+^ lymphocytes within the LP (Figure 3). Isolated LP cells were cultured in *vitro* for 24 hours and spontaneous secretion of T~17~ polarizing cytokines were measured in culture supernatants. Both IL-18 and IL-6 secretion were significantly reduced by *B. infantis* feeding. Citral reversed the suppression of IL-18 secretion and partially reversed the suppression of IL-6 secretion (Figure 3). One potential explanation for the *B. infantis* suppression of T~17~ cells within the LP is that *B. infantis* may alter lymphocyte recruitment to the LP. Expression of the intergrin Tαβ7 and the chemokine receptor CCR9 influence the gut homing of lymphocytes. Both Tαβ7^+^ and CCR9^+^ lymphocytes populations were significantly suppressed following *B. infantis* feeding (Figure 3). However, citral treatment had no effect on the *B. infantis* suppression of Tαβ7^+^ lymphocytes, with a minor effect on CCR9^+^ lymphocytes, suggesting that the *B. infantis* effect on lymphocyte homing is independent of retinoic acid metabolism.

*B. infantis* induction of Foxp3^+^ lymphocytes is retinoic acid-independent

As previously described, *B. infantis* feeding was associated with increased numbers of regulatory lymphocytes within the mucosa (Figure 4). Foxp3^+^ T lymphocytes were increased within the LP and showed a tendency to be increased for MLN (Figure 4). However, citral did not attenuate the increase in Foxp3^+^ lymphocytes suggesting that mechanisms other than retinoic acid metabolism can be responsible for this effect. IL-10^+^ CD4^+^ lymphocytes were significantly increased in the MLN, but not the LP of *B. infantis*-fed animals, which was blocked by citral (Figure 4).

*B. infantis* attenuation of colitis is retinoic acid-dependent

In the DSS model of colitis, *B. infantis* feeding was associated with decreased histopathology (Figure 5a and 5b) and decreased MPO levels (Figure 5c). Citral treatment blocked the protective effect of *B. infantis* feeding, which suggests that mechanisms other than retinoic acid metabolism can be responsible for this effect.

Figure 1. *B. infantis* is sampled by dendritic cells within PP and LP. (a) Flow cytometric analysis of CD11c^+^MHCI^+^ dendritic cells within the PP and LP at 0 or 2 hours following gavage of CFSE-labelled *B. infantis*, revealed that a subpopulation of dendritic cells at both sites become CFSE^+^. (b) Visualization by multispectral flow cytometry imaging confirmed the presence of CFSE-labelled bacteria on PP dendritic cells at 2 hours.

doi: 10.1371/journal.pone.0062617.g001

Immune Regulation by Retinoic Acid
The effect of *B. infantis* feeding. Citral itself did not impact the severity of DSS-induced colitis (Figure 5a) or LP TH1 and TH17 subsets (Figure 6a). However, *B. infantis* significantly reduced the numbers of IL-17+ and IFN-γ+ lymphocytes within the LP of colitic animals, which was blocked by citral treatment (Figure 6a). The proportion of Foxp3+ lymphocytes within the MLN increased significantly due to the induction of colitis and *B. infantis* feeding had no effect on the increase in Foxp3+ lymphocytes within the MLN (Figure 6b). In contrast, DSS-induced colitis was associated with significantly increased numbers of CD103+ dendritic cells within the LP (Figure 7a). CD103+RALDH+ dendritic cells are elevated in the LP following *B. infantis* feeding. Flow cytometric assessment of LP and MLN revealed that *B. infantis* feeding is associated with increased CD103+RALDH+ dendritic cells within the LP (n = 7), compared to the control group (n = 8), analysed using unpaired student t-tests (a). Citral blocked the increase in LP CD103+RALDH+ dendritic cells (n = 7). (b) Multispectral flow cytometry imaging identified CD103+ dendritic cells that efficiently bind CFSE-labelled *B. infantis*. (c) Flow cytometric analysis of CD11c+MHCII+CD103+ dendritic cells from the mucosa demonstrated that approximately 38% of CD103+ dendritic cells bound *B. infantis*. Isolated mucosal CD11c+ dendritic cells upregulate mRNA for RALDH enzymes following *in vitro* incubation with *B. infantis* (d), while the increase in gene expression is specific to CD103+ dendritic cells (e). doi:10.1371/journal.pone.0062617.g002
Figure 3. *B. infantis* alters lymphocyte phenotypes within the LP. (a) IL-17$^+$ lymphocytes were significantly reduced and IFN-gamma$^+$ cells were substantially reduced within the LP of *B. infantis*-fed mice (n = 8), compared to the control group (n = 6), an effect that was blocked by citral (n = 5). No effect was observed for IL-4$^+$ lymphocytes. (b) Isolated LP was cultured *in vitro* and cytokine secretion measured after 24 hours. *B. infantis* feeding reduced the *in vitro* secretion of the TH17-polarising cytokines IL-1$\beta$ and IL-6, which was partially reversed by citral. (c) *B. infantis* feeding was associated with a decrease in the proportion of LP lymphocytes expressing the gut homing receptors $\alpha 4\beta 7$ and CCR9. Citral did not reverse the decrease in $\alpha 4\beta 7^+$ lymphocytes and had a minor influence on CCR9$^+$ lymphocytes. Statistical significance was estimated using unpaired student t-tests.

doi:10.1371/journal.pone.0062617.g003
Figure 4. *B. infantis* induction of Foxp3+ lymphocytes is not RALDH-dependent. (a) Representative flow cytometric dot-plots are illustrated for CD4 and Foxp3 populations within MLN and LP. (b) The increase in CD4+Foxp3+ T lymphocytes following *B. infantis* feeding in the LP (n = 6) is not reversed by citral treatment (n = 5). (c) CD4+IL-10+ T lymphocytes increased within MLN (n = 8), but not the LP (n = 7). LP statistical significance was estimated using the non-parametric Mann-Whitney test, while MLN statistics were determined using the parametric unpaired student t-test. doi:10.1371/journal.pone.0062617.g004
suppression of RALDH^+ CD103^+ dendritic cells (Figure 7b). Moreover, the frequency of CD11c^+ CD103^+ RALDH^+ cells was reduced in the inflamed LP and *B. infantis* feeding did not affect retinoic acid production by CD103^- cells (Figure 7c). The upregulation of CD103 in the colitis group was primarily seen on CD11c^+CD11b^- dendritic cells, while the upregulation of CD103 in the *B. infantis* group was within the CD11c^+CD11b^- dendritic cell subpopulation (Figure 7d).

**Discussion**

In this study, we show that *B. infantis* is sampled by mucosal dendritic cells within the LP and PP, resulting in increased numbers of LP CD103^+RalDH^+ dendritic cells with tolerogenic properties. The suppression of TH1 and TH17 lymphocytes within the LP was observed in the healthy and inflamed gut, which was dependent on retinoic acid metabolism as citral administration blocked this activity. In addition, disease severity was reduced by *B. infantis* feeding during DSS-induced colitis, which was also blocked by citral. Interestingly the elevation in mucosal LP Foxp3

![Histology Score](image)

**Figure 5.** DSS-induced colitis is reduced by *B. infantis* feeding. Four murine study groups were examined – Untreated (no DSS and no *B. infantis*), Control (DSS alone), *B. infantis* (DSS and *B. infantis*), citral (DSS and citral) and *B. infantis* & citral (DSS, *B. infantis* and citral). (a) The histopathology inflammatory score was significantly reduced by *B. infantis* feeding (n = 5), but not when citral was co-administered (n = 8). Citral administered with DSS did not increase inflammation in the colon in comparison to DSS alone (n = 7). (b) Representative slides of the murine gut are illustrated. (c) Colonic myeloperoxidase (MPO) levels were reduced in *B. infantis*-fed mice, which was not observed with *B. infantis* and citral treatment. Statistical significance was determined using non-parametric Mann-Whitney tests.

doi:10.1371/journal.pone.0062617.g005
lymphocytes, but not MLN IL-10⁺ lymphocytes, associated with B. infantis feeding was RALDH-independent.

LP CD103⁺ dendritic cells are derived from circulating common dendritic cell precursors (not from LP CD103⁻ intermediates), require Flt3 ligand for their development and migrate efficiently to the draining lymph nodes [22,23]. However, the microbial factors that influence the tolerogenic potency of CD103⁺ dendritic cells within the LP are only beginning to be elucidated. One study using germ-free animals suggested that CD103⁺ dendritic cells within the colon did not require the presence of a microbiota [24]. In contrast, another study recently demonstrated that Bifidobacterium breve promoted development of IL-10-producing Tr1 cells in the colon by intestinal CD103⁺ dendritic cells via the TLR2/MyD88-dependent induction of IL-27 and IL-10 [25]. We have also previously demonstrated in vitro that B. infantis-induced IL-10 secretion by human myeloid dendritic cells was TLR2-dependent [21]. However, Jeon et al did not detect alterations in T_{h}17 or T_{h}1 populations within the colon following B. breve feeding [25]. As they did not measure CD103⁺RALDH activity, direct comparisons with the present study are not possible but it’s clear that the B. infantis-associated suppression of T_{h}17 and T_{h}1 cells is dependent on RALDH activity.

Intestinal homeostasis is maintained by regulatory T cell populations consisting of two major CD4⁺ T cell subsets, Foxp3⁺ T_{reg} cells and IL-10-producing Tr1 cells [26]. Site-specific alterations in regulatory lymphocyte subsets are evident in this study. Within the LP, B. infantis increased the proportion of

Figure 6. B. infantis alters T cell phenotypes within the inflamed LP. (a) DSS colitis increases the proportion of IL-17⁺ and IFN-gamma⁺ CD4 T lymphocytes within the LP (n = 8). However, B. infantis feeding (n = 6) significantly reduces LP IL-17⁺ and IFN-gamma⁺ subpopulations compared to DSS alone, which was inhibited by citral (n = 7). Both subpopulations were similarly increased in DSS alone and DSS & citral (n = 7). (b) CD4⁺Foxp3⁺ lymphocytes were significantly increased in the MLN during DSS colitis (n = 6), with a significant decrease of Foxp3⁺ lymphocytes being observed within the LP (n = 6). B. infantis feeding (n = 6) did not alter the increase in Foxp3⁺ cells in the MLN, but partially restored the deficit in Foxp3⁺ cells within the LP (n = 6). (c) Expression of the gut homing receptors α4β7 and CCR9 did not significantly change for any of the groups examined. Statistical significance was determined using unpaired student t-tests.

doi:10.1371/journal.pone.0062617.g006
Foxp3⁺ lymphocytes, while Tr1 cells were increased only within the MLN. Alternative mechanisms also are required for induction of the two regulatory populations as citral blocked the induction of MLN Tr1 cells, but not the elevation in Foxp3⁺ cells within the LP. Suppression of T_{H1} and T_{H17} lymphocytes within the LP was abrogated by citral confirming that citral administration did have an effect within the LP. Thus, the *B. infantis* induction of Foxp3⁺ lymphocytes within the LP involves mechanisms other than retinoic acid metabolism. Similarly, *B. infantis* reduced the proportion of α4β7 and CCR9 lymphocytes within the LP, which was not dramatically influenced by citral. Retinoic acid has been previously described to upregulate expression of gut homing receptors, which was not observed in our studies. This finding further supports the existence of additional non-RALDH-dependent mechanisms which are induced by *B. infantis* within the LP [27].

Even though RALDH and non-RALDH mechanisms may be required for *B. infantis*-associated immunoregulatory activity within the mucosa, citral blocked the anti-inflammatory effect of *B. infantis* in the DSS colitis model confirming that the induction of retinoic acid is critical for the immunoregulatory properties of *B. infantis*.
acid metabolism is critical for the in vivo protective effects of this microbe. Similar to the findings with healthy mice, B. infantis reduced T411 and T417 lymphocytes within the inflamed LP, which was retinoic acid-dependent. In contrast to healthy mice, B. infantis did not reduce the number of lymphocytes expressing γδT and CCR9 within the inflamed LP. While certain therapeutic approaches have focussed on blocking gut homing receptors for amelioration of colitis, it has also been shown that CCR9 deficiency exacerbates colitis due to impairment of Treg recruitment to the gut [28,29]. However, our study suggests that B. infantis does not alter recruitment of γδT or CCR9 positive lymphocytes into the inflamed LP.

During DSS-induced colitis, the relative proportion of CD11b+CD103+ dendritic cells was increased within the LP, while B. infantis feeding was associated with an increase in CD11b+CD103+ dendritic cells. The LP CD11b+CD103+ dendritic cell subset has been suggested to possess proinflammatory properties within the inflamed gut [11,30]. In addition, this dendritic cell population was shown to play a key role in T417 cell differentiation in vitro [31]. Furthermore, CD11b+CD103+ dendritic cells express TLR5 at a high level, rapidly respond to flagellin stimulation resulting in IL-23 secretion and are very efficient in presenting antigens to CD4+ lymphocytes [12,32,33]. Our data suggests that B. infantis, even within an inflamed microenvironment, continues to induce regulatory CD11b+CD103+’RALDH+’ dendritic cells within the LP and suppresses the increase in the proinflammatory CD11b+CD103+’ dendritic cell population. Interestingly, the suppression of T417 cells within the inflamed LP of B. infantis-fed mice correlates with the suppression of the CD11b+CD103+’ dendritic cell subset. Further investigation is required to determine if there is a direct connection between B. infantis associated suppression of CD11b+CD103+’ dendritic cells and T417 polarization.

Within the mucosa, dendritic cells are integral to promoting oral tolerance and preventing pathological immune responses to harmless antigens. Dendritic cells use signals derived from their local environment to shape the development of low-level immune responses to the commensal microbiota, which controls the microbiota without causing pathology. The breakdown in dendritic cell regulatory networks is associated with aberrant inflammatory activity within the gut and therapeutic strategies aimed at re-establishing dendritic cell tolerogenic tone would be of benefit to IBD, IBS and food allergy patients. One such strategy is the deliberate manipulation of CD103+’RALDH+’ dendritic cells by microbes or microbial components in combination with dietary supplementation with vitamin A.

The murine data presented in this report strongly support the further evaluation of these strategies in human clinical studies.

Supporting Information

Figure S1 B. infantis is bound at a low frequency by CD11c+MHCIId+CD103+ dendritic cells. Flow cytometric analysis of CD11c+MHCIId+CD103+ dendritic cells from the mucosa demonstrated that approximately 10% of CD103+ dendritic cells bound B. infantis. (PPT)

Author Contributions

Conceived and designed the experiments: CAA RPL LO. Performed the experiments: PK R. Fersl MZ R. Frei DN. Analyzed the data: PK R. Frei R. Fersl DN LO. Contributed reagents/materials/analysis tools: CAA RPL LO. Wrote the paper: PK CAA LO.

References

1. Shanahan F (2010) Gut microbes: from bugs to drugs. Am J Gastroenterol 105: 275–279.
2. Leavy O (2012) Mucosal immunology: the good the gut bugs do. Nat Rev Immunol 13: 319.
3. Lee YK, Mazmanian SK (2010) Has the microbiota played a critical role in the evolution of the adaptive immune system? Science 330: 1768–1773.
4. Frei R, Launep RP, Craven K, O’Malley L (2012) Microbiota and dietary interactions: an update to the hygiene hypothesis? Allergy 67: 451–461.
5. Feng T, Elson CO (2011) Adaptive immunity in the host-microbiota dialog. Immunity 34: 414–428.
6. Taylor BC, Zaph C, Troy AE, Du Y, Guild KJ, et al. (2009) TSLP regulates intestinal immunity and inflammation in mouse models of helminth infection and colitis. J Exp Med 206: 655–667.
7. Coombes JL, Siddiqui KR, Arancibia-Cárovo CV, Hall J, Sun CM, et al. (2007) A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med 204: 1757–1769.
8. Sun CM, Hall JA, Blank RB, Bouladoux N, Oudka M, et al. (2007) Small intestine lamina propria dendritic cells promote de novo generation of Foxp3+ T reg cells via retinoic acid. J Exp Med 204: 1773–1785.
9. Maricassamy S, Paleodrak B (2009) Retinoic acid-dependent regulation of immune responses by dendritic cells and macrophages. Semin Immunol 21: 22–27.
10. Laffont S, Siddiqui KR, Powrie F (2010) Intestinal inflammation abrogates the tolerogenic properties of MLN CD103+ dendritic cells. Eur J Immunol 40: 1877–1883.
11. Uematsu S, Fujimoto K, Jang MH, Yang BG, Jung YJ, et al. (2008) Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. Nat Immunol 9: 769–776.
12. Manicassamy S, Ravindran D, Deng J, Oltcho H, Denning TL, et al. (2009) Toll-like receptor 2-dependent induction of vitamin A-metabolizing enzymes in dendritic cells promotes T regulatory responses and inhibits autoimmunity. Nat Med 15: 401–409.
13. Takahashi H, Kanno T, Nakayamada S, Hirakura K, Sciamé G, et al. (2012) TGF-β and retinoic acid induce the microRNA miR-10a, which targets Bcl-6 and inhibits the helper cell T pool. Nat Immunol 13: 587–595.
28. Murphy CT, Moloney G, Macsharry J, Haynes A, Faivre E, et al. (2010) Function and efficacy of an [alpha]4-integrin antagonist using bioluminescence imaging to detect leukocyte trafficking in murine experimental colitis. J Leukoc Biol 88: 1271–1278.

29. Wermers JD, McNamee EN, Wurbel MA, Jellicka P, Rivera-Nieves J (2011) The chemokine receptor CCR9 is required for the T-cell-mediated regulation of chronic ileitis in mice. Gastroenterology 140: 1526–1535.

30. Manicasamy S, Pulendran B (2011) Dendritic cell control of tolerogenic responses. Immunol Rev 241: 206–227.

31. Denning TL, Norris BA, Medina-Contreras O, Manicasamy S, Geem D, et al. (2011) Functional specializations of intestinal dendritic cell and macrophage subsets that control Th17 and regulatory T cell response are dependent on the T cell/APC ratio, source of mouse strain, and regional localization. J Immunol 187: 733–747.

32. Kinnebrew MA, Buffie CG, Diehl GE, Zenewicz LA, Leiner I, et al. (2012) Interleukin 23 production by intestinal CD103(+)CD11b(+) dendritic cells in response to bacterial flagellin enhances mucosal innate immune defense. Immunity 36: 276–287.

33. Rivollier A, He J, Kole A, Valatas V, Kelsall BL (2012) Inflammation switches the differentiation program of Ly6Chi monocytes from antiinflammatory macrophages to inflammatory dendritic cells in the colon. J Exp Med 209: 139–155.