

Selected commensal-related bacteria and Toll-like receptor 3 agonist combinatorial codes synergistically induce interleukin-12 production by dendritic cells to trigger a T helper type 1 polarizing programme

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Summary

Enteric infections remain a major health problem causing millions of deaths in developing countries. The interplay among the host intestinal epithelium, the mucosa-associated immune system and microbiota performs an essential role in gut homeostasis and protection against infectious diseases. Dendritic cells (DCs) play a key role in orchestrating protective immunity and tolerance in the gut. The mechanisms by which DCs adapt their responses and discriminate between virulent microbes and trillions of innocuous bacteria remain ill-defined. Here we investigated the effect of cross-talk between commensal-related bacteria (CB) and Toll-like receptor (TLR) agonists on DC activation and the outcome of the *in vitro* T helper response. Human monocyte-derived DCs were exposed to eight different Gram-positive or Gram-negative CB strains prior to activation with five different TLR agonists. The key polarizing cytokines interleukin (IL)-12p70, IL-10, IL-1 β and IL-6 were quantified and the fate of naïve T-cell differentiation was evaluated. We identified a unique combination of *Lactobacillus casei* and TLR3 signals that acted in synergy to selectively increase IL-12p70 secretion. Exposure to poly(I:C) converted *L. casei*-treated DCs into potent promoters of T helper type 1 (Th1) responses. We propose that DCs can integrate harmless and dangerous non-self signals delivered by viral products, to mount robust Th1 responses. Thus, *in vivo* DC targeting with selective probiotics may improve strategies for the management of enteric diseases.

Keywords: commensal bacteria; dendritic cells; interleukin-12; T helper type 1; Toll-like receptor

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Introduction

In the gastrointestinal tract (GT), about 10¹⁴ commensal bacteria encompassing 300–500 different strains have developed a symbiotic relationship with mammalian hosts.¹ The human microbiome encodes physiological components (for instance enzymes) that complement host capacities or deficiencies.² The mucosal immune system needs to be tightly regulated to avoid collateral damage to self and food antigens as well as to innocuous bacteria of the commensal flora. Remarkably, it maintains the capacity to mount a powerful defence response against pathogens. In the gut mucosa, non-pathogenic bacteria are constantly in contact with epithelial cells and dendritic cells (DCs). DCs extend dendrites across the epithelial barrier and express molecules that participate in the

formation of tight junctions.³ Intestinal bacteria can be recognized by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) expressed by epithelial cells.⁴ The loss of gut epithelium integrity in MyD88-deficient mice after chemical exposure underscores the importance of this type of interaction.⁵

Such an equilibrium established by permanent contact with commensal flora enforces gut tolerance which is disturbed by the introduction of pathogens. Direct DC recognition of viral and microbial products [pathogen-associated molecular patterns (PAMPs)] by PRRs that include TLRs and NOD-like receptors (NLDs), together with inflammatory cytokine release, is mandatory to drive and sustain effector Th cell responses.^{6,7} DCs are at the interface between innate and adaptive immunity and it is now clear that they direct different classes of immune

responses.^{8,9} DCs can be designated as tolerogenic or immunogenic because they can elicit tolerance or T-cell immunity. IL-12 and IL-10 produced by DCs dictate CD4⁺ T-cell fate, inducing differentiation into pro-inflammatory (Th1) and regulatory (Tr1) T cells, respectively. Transforming growth factor (TGF)- β is critical for the differentiation of adaptive forkhead box P3 (FoxP3) regulatory T cells (Tregs) but, together with IL-6, it promotes the generation of IL-17-producing cells in mice.¹⁰ In contrast, IL-1 β or IL-21 promotes human Th17 development.^{11,12}

Direct interaction between commensal-related bacteria (CB) and DCs may also occur *in vivo*,¹³ although the precise nature of the signals delivered remains ill-defined. *Bifidobacteria* prime human DCs via TLR2, while *Lactobacilli* do not signal through TLR1, TLR2, TLR3, TLR4, TLR6, TLR7 or TLR9.^{14,15} We¹⁶ and others¹⁷ have reported that Gram-positive and Gram-negative CB induce phenotypic and functional DC maturation. Yet, CB-treated mature DCs convert naïve CD4⁺ T cells into suppressor T cells in the absence of a TLR agonist signal.¹⁶ As microbe-associated molecular patterns are shared by commensals and pathogens, how the DCs sense harmless commensals and dangerous pathogens remains an important question to answer.

In the present study, we hypothesize that DCs may integrate signals received simultaneously or consecutively from CB and pathogens. Indeed, recent studies have validated the concept that synergy among multiple TLR signals sustains human Th1 responses.¹⁸ We thus postulate that CB impinge on gut tolerance at steady state by inducing suppressor CD4⁺ T cells and co-operate with selected pathogens to drive protective Th effector responses. We thus sought to investigate the effect of cross-talk between Gram-positive or Gram-negative CB and TLR agonists on monocyte-derived DC function and on the development of CD4⁺ T helper responses. Monocytes are the precursors of mucosal DCs *in vivo*; when recruited in large numbers at inflamed sites, they differentiate into DCs.¹⁹ Among eight different CB strains and five TLR agonists examined, we identified one combinatorial code, i.e. *Lactobacillus casei* and the TLR3 signal, which synergized to selectively increase IL-12p70 production by DCs and drive naïve CD4⁺ T-cell polarization towards a strong Th1 response without inducing Th17. We here propose a mechanism by which harmless non-self (i.e. CB) and dangerous (i.e. virus) signals may combine to shift DC function from tolerogenic to immunogenic and perhaps elicit protective immunity.

Materials and methods

Reagents

Eight gamma-irradiated CB strains (*L. casei* DN-114 001, *L. casei* no.2 DN-114 086, *Lactobacillus rhamnosus* DN-116

047, *Lactobacillus plantarum* DN-121 022, *Bifidobacterium animalis* DN-173 010, *Bifidobacterium adolescentis* DN-150 017, *Streptococcus thermophilus* DN-001 621 and *Bacteroides thetaiotaomicron*, a Gram-negative commensal-related species) were used in the cultures at a CB:DC ratio of 200 : 1 unless indicated otherwise. *Bifidobacteria* and *Bacteroides* are representative of the dominant species of human microflora, whereas *Lactobacilli* and *Streptococci* are representative of subdominant human flora. The irradiation process prevented bacterial overgrowth in cell cultures and maintained bacterial structural integrity. A similar DC cytokine profile (IL-12:IL-10 ratio) was obtained with live bacteria and 200 : 1 was determined to be the optimal CB:DC ratio for DC phenotypic maturation.¹⁶ Cytokines and chemokines were measured in culture supernatants (CSNs) using commercially available enzyme-linked immunosorbent assay (ELISA) kits for IL-1 β , IL-10, IL-12p70 and IL-17 (R&D Systems, Minneapolis, MN), interferon (IFN)- γ and IL-6 (BioSource, San Diego, CA), IL-23 (eBioscience, San Diego, CA) and chemokine (C-X-C motif) ligand 10 (CXCL10) (BD Biosciences, San Jose, CA). TLR agonists were used at the following concentrations: 100 μ g/ml for peptidoglycan (PGN) (Sigma-Aldrich, St Louis, MO), 15 μ g/ml for poly(I:C) (GE Health Sciences, Buckinghamshire, UK), 10 μ g/ml for lipopolysaccharide (LPS) (Sigma-Aldrich), 20 μ g/ml for flagellin, and 8 μ g/ml for single-stranded RNA (ssRNA) (InvivoGen, San Diego, CA). Resveratrol was purchased from Sigma-Aldrich and used at a final concentration of 50 μ M.

Cell preparation, purification and culture conditions

Human peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation of heparinized blood from health volunteers using Lymphoprep (Axis-Shield PoS AC, Dundee, UK). Permission to use human primary cells for the described experiments was obtained from the Ethic Research Committee of the Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM). Monocyte-derived DCs were prepared from enriched monocytes as described by Demeure *et al.*²⁰, following 5–6 days of culture in the presence of 25 ng/ml of IL-4 and 25 ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) (R&D Systems). Naïve CD4⁺ T cells were isolated using the Human CD4⁺ Naïve T-cell Enrichment Kit (StemCell Technologies, Vancouver, Canada) according to the protocol provided by the manufacturer.

Immature DCs (iDCs) were cultured with CB alone or in combination with TLR agonists in HB101 complete medium (Irvine Scientific, Santa Ana, CA) for 48 hr at the indicated CB:DC ratio. Naïve CD4⁺ T cells were co-cultured for 5–7 days with mature DCs at a DC:T-cell ratio of 1 : 10 in RPMI-1640 medium (Wisent Inc., St-Bruno, Canada) supplemented with 10% fetal calf

serum (FCS) (Gibco–Invitrogen, Carlsbad, CA). For some experiments, T cells were cultured for 5 days with plate-bound anti-CD3 (10 mg/ml; OKT3; Janssen-Ortho, Tronto, Canada) in Yssel's medium (Gemini Bio-product, West-Sacramento, CA) supplemented with 2% human AB serum (Wisent) in the presence of filtered culture supernatants of activated DCs. Primed T cells were expanded for 3–7 days in the presence of IL-2 (50 U/ml) (R&D Systems). In selected experiments, neutralizing monoclonal antibody (mAb) to IL-12 (clone C8.6.22 or clone 24910, specific to IL-12p70; R&D Systems) or isotype-matched control (10 µg/ml) was added to T-cell primary cultures.

To promote Th17 differentiation, naïve CD4⁺ T cells were stimulated on plate-bound anti-CD3 in Yssel's medium supplemented with 2% human AB serum in the presence of anti-IFN- γ (1.5 µg/ml), IL-23 (10 ng/ml) and IL-1 (10 ng/ml) (R&D Systems) with or without IL-12 (60 pM) (F. Hoffman-La Roche, Basel, Switzerland) for 5 days. Cells were expanded for 5 days in the presence of IL-2 (20 U/ml).

In vitro suppression assay

To evaluate regulatory function, freshly isolated CD4⁺ T cells (2×10^5 cells/ml) were cultured alone (target cell \times 1) or together with primed T cells at a 1 : 3 effector:target cell ratio and stimulated for 3–5 days with anti-CD3 (OKT3) (100 ng/ml) plus anti-CD28 (500 ng/ml) (BD Biosciences) in the presence of mitomycin C-treated monocytes (2×10^4 cells/ml). Proliferation of CD3-activated CD4⁺ T cells alone (target cell \times 1) and proliferation of CD4⁺ T cells mixed at a cell ratio of 1 : 3 (target cell \times 2) were used as reference values to evaluate the suppressive function of primed T cells. Cell proliferation was assessed using [³H]thymidine (GE Health Sciences) incorporation.

Antibodies and flow cytometry analysis

For intracellular cytokine staining, primed T cells were re-stimulated with 10 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich), 1 µg/ml of ionomycin (Calbiochem-Behring, San Diego, CA) and 3 µM of monensin (Calbiochem-Behring) for 6 hr. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) and permeabilized with 0.1% saponin. Cells were stained for intracellular IFN- γ , IL-4 (BD Biosciences) and T-bet (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical analysis

Statistical analysis was performed using the two-tailed Student's *t*-test assuming equal variance between the compared groups, or a one-way analysis of variance (ANOVA) for multiple groups. Statistical significance is indicated in the figures (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

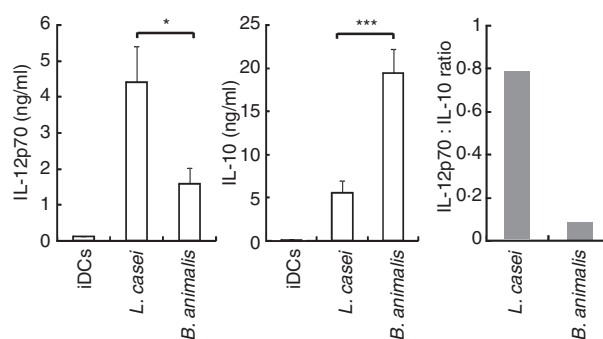


Figure 1. Cytokine profiles of dendritic cells (DCs) stimulated with *Lactobacillus casei* and *Bifidobacterium animalis*. Interleukin (IL)-12p70 and IL-10 secretion and the IL-12p70:IL-10 ratio in the 48-hr culture supernatants (CSNs) of DCs stimulated in the absence [immature DCs (iDCs)] or the presence of *L. casei* or *B. animalis* (bacteria : DC ratio at 200 : 1) were determined. The mean \pm standard error of the mean (SEM) for 20 independent experiments is shown. Student's *t*-test was performed: *** $P < 0.001$; * $P < 0.05$.

Results

The unique combination of *L. casei* plus poly(I:C) selectively enhances IL-12p70 production

Human monocyte-derived DCs sustain IL-12p70 production *in vitro* solely in response to the activation of TRIF-coupled receptors (TLR3 or TLR4) combined with a delayed or concomitant endosomal receptor (TLR7, TLR8 or TLR9) signal.^{18,21} We here investigated whether direct exposure of DCs to a TLR signal combined with CB augmented IL-12p70 production. We first selected *L. casei* and *B. animalis* CB strains as they are representative of two major groups of CB strains that can be distinguished by their contrasting IL-12:IL-10 cytokine ratios (Fig. 1). *Lactobacillus casei* (Fig. 2a) but not *B. animalis* (Fig. 2b) added to DCs 4 hr prior to poly(I:C) (TLR3 ligand) resulted in a fivefold increase in IL-12p70 secretion as compared with bacteria or TLR alone. A small but significant enhancing effect was also observed when *L. casei* was combined with LPS (TLR4 ligand) (Fig. 2a). Addition of IFN- γ further augmented *L. casei* plus poly(I:C)-induced IL-12p70 production (data not shown). The *L. casei*/poly(I:C) combination was rather selective for IL-12p70 in as much as it did not facilitate IL-10, CXCL10 or IL-1 β release and only slightly enhanced that of IL-6 (*L. casei*/TLR agonist versus *L. casei* or TLR agonist alone) (Fig. 2a and Fig. S1a). Flagellin (TLR5 ligand), PGN (TLR2 or NOD ligand) and ssRNA (TLR8) triggered cytokine release when used alone but did not augment *L. casei*-induced release of IL-12p70, IL-10, CXCL10, IL-1 β or IL-6 (Fig. 2a and Fig. S1a). In contrast, none of the TLR agonists modulated the IL-10 secretion induced by *B. animalis*-treated DCs (Fig. 2b). As reported by Napolitani *et al.*,¹⁸ we confirmed that addition of poly(I:C)

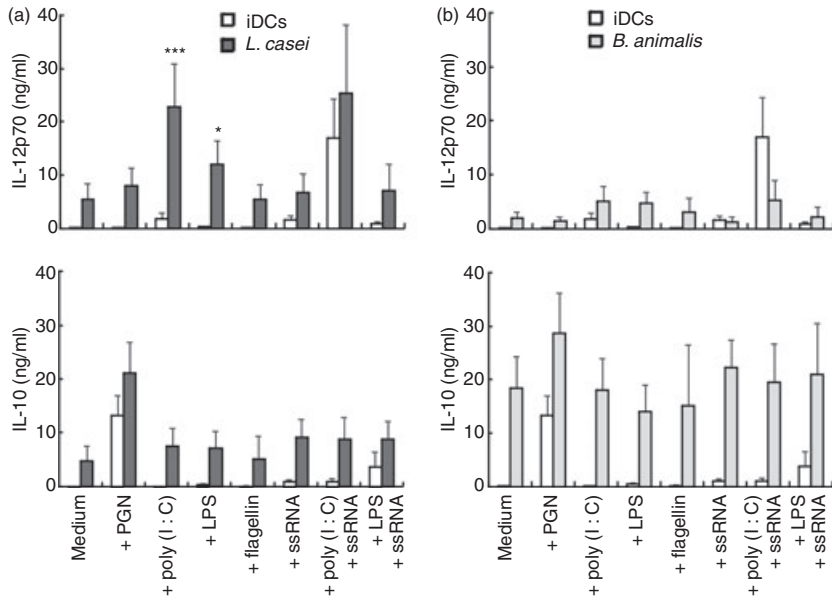


Figure 2. Cytokine profiles of dendritic cells (DCs) stimulated with *Lactobacillus casei* or *Bifidobacterium animalis* in combination with various Toll-like receptor (TLR) agonists. Interleukin (IL)-12p70 and IL-10 production in 48-hr culture supernatants (CSNs) was determined. DCs were stimulated with *L. casei* (a) or *B. animalis* (b) 4 hr prior to the addition of medium or TLR agonists [peptidoglycan (PGN), poly(I:C), lipopolysaccharide (LPS), flagellin, and single-stranded RNA (ssRNA) alone and combined with poly(I:C) or LPS]. The mean \pm standard error of the mean (SEM) for three to 18 independent experiments is shown. In the analysis of variance (ANOVA), the effect of commensal-related bacteria (CB) plus TLR agonists was compared with that of a single agent, i.e. CB or TLR agonists alone: *** $P < 0.001$; * $P < 0.05$.

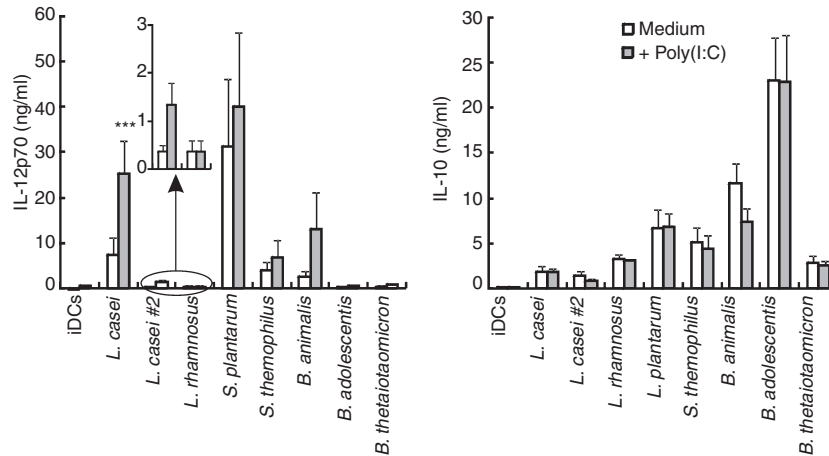


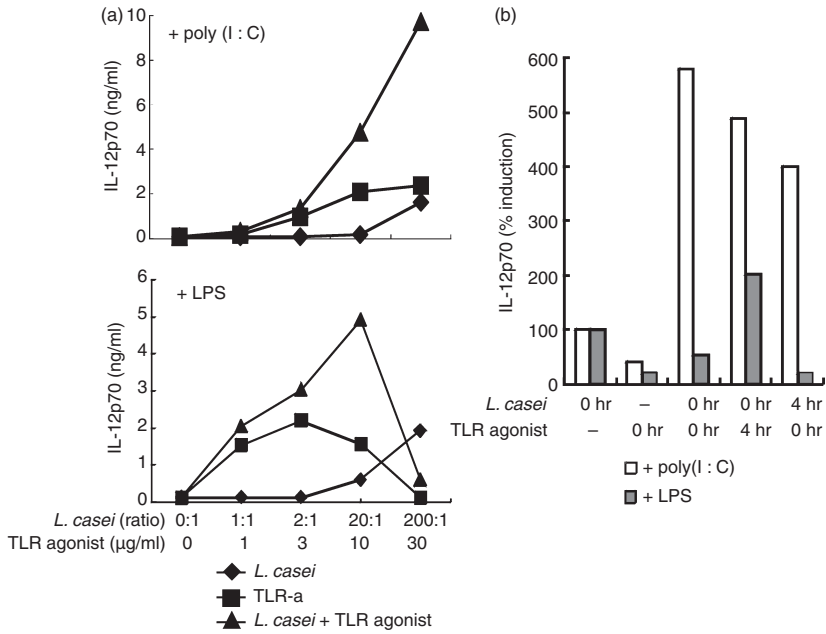
Figure 3. Cytokine profiles of dendritic cells (DCs) stimulated with poly(I:C) in combination with various strains of commensal-related bacteria (CB). Interleukin (IL)-12p70 and IL-10 release in 48-hr culture supernatants (CSNs) of DCs stimulated in the absence or presence of *Lactobacillus casei*, *Lactobacillus casei* no.2, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Streptococcus thermophilus*, *Bifidobacterium animalis*, *Bifidobacterium adolescentis* and *Bacteroides thetaiotaomicron*, a Gram-negative CB, 4 hr prior to exposure to medium or poly(I:C). The mean \pm standard error of the mean (SEM) for five independent experiments is shown. *** $P < 0.001$ (analysis of variance). iDC, immature dendritic cell.

4 hr prior to ssRNA strongly increased IL-12p70 production, which was not further augmented by *L. casei*. Six additional CB were examined and could not substitute for *L. casei* to enhance poly(I:C) or LPS-induced IL-12p70 production, nor did they facilitate IL-10, IL-6 or IL-1 β production (Fig. 3 and Fig. S2). These included two potent IL-12 inducers (*L. plantarum* and *S. thermophilus*), an IL-10 inducer (*B. adolescentis*) and a weak IL-10 and IL-12 inducer (the Gram-negative *B. thetaiotaomicron*). However, a small increase in IL-12p70 secretion was noted with the *L. casei* no.2 (DN-114 086 strain)/poly(I:C) combination. As reported by others,²² our data revealed that *L. rhamnosus* and *L. plantarum* decreased LPS-induced IL-10 and IL-6 secretion.

***Lactobacillus casei* synergizes with TLR3 signals to selectively enhance IL-12p70 production by DCs**

We next explored whether this unique signalling through TLR3/TLR4 and *L. casei* exerted a synergistic or an additive effect on IL-12p70 production (Fig. 4a). In support of a synergistic mode of action, a combination of sub-optimal doses of poly(I:C) or LPS plus *L. casei* induced IL-12p70 production at concentrations that were higher than those induced by optimal doses of a single agonist. LPS at 30 μ g/ml alone or combined with *L. casei* (200 : 1) decreased cytokine production and cellular viability. We next investigated the window of time in which *L. casei* and TLR displayed synergistic properties

Figure 4. Dose–response and temporal requirements of *Lactobacillus casei*/Toll-like receptor (TLR) agonist combinatorial stimulation. (a) Interleukin (IL)-12p70 production by dendritic cells (DCs) stimulated with a range of concentrations (as indicated on the x-axis of the figure) of *L. casei*, poly(I:C), lipopolysaccharide (LPS) or combinations of *L. casei* plus TLR agonists. (b) Temporal requirement for *L. casei*/poly(I:C) or LPS synergy. TLR agonists were added to DC cultures simultaneously 4 hr after (0 hr, 4 hr) or 4 hr prior to (4 hr, 0 hr) addition of *L. casei*. IL-12p70 is shown as the percentage induction compared with *L. casei* alone. One representative experiment of three is shown.

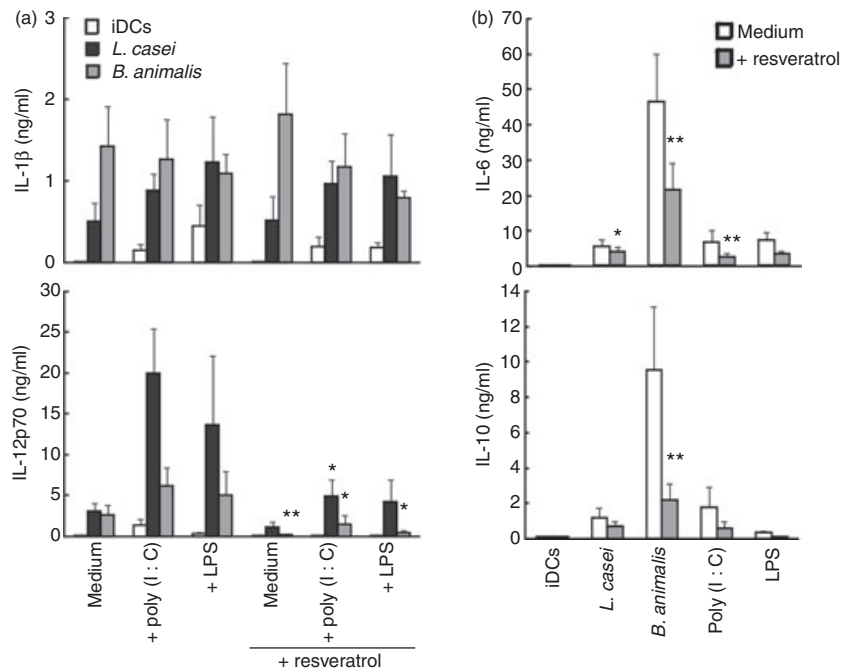


(Fig. 4b). *Lactobacillus casei* added simultaneously, 4 hr prior to or after poly(I:C), increased IL-12p70 production. In contrast, DCs absolutely required to be exposed to *L. casei* 4 hr prior to the addition of LPS to produce significant amounts of IL-12p70; the addition of LPS before *L. casei* rendered DCs refractory to LPS stimulation of IL-12p70 production and that of other cytokines.

The signalling pathway used by CB to activate human DCs is still a matter of debate. *Lactobacilli* are unable to

activate any of the TLR family, whereas culture supernatant of *B. breve* activates the TLR2 pathway.^{14,15} Here we examined whether resveratrol, an inhibitor of TRIF-coupled receptor and TLR2 signalling,^{23,24} interfered with IL-12 release induced by *L. casei*, *L. casei* plus TLR3 or *L. casei* plus TLR4 (Fig. 5a). Notably, resveratrol abolished poly(I:C)- or LPS-induced IL-12p70 production and significantly decreased the *L. casei*/poly(I:C) combinatorial-induced IL-12p70 production to concentrations equivalent to those induced by *L. casei* alone. IL-1β

Figure 5. *Lactobacillus casei*/poly(I:C) or lipopolysaccharide (LPS) synergy in the production of interleukin (IL)-12p70 is inhibited by resveratrol. (a) IL-1β and IL-12p70 secretion in culture supernatants (CSNs) of DCs stimulated in the absence or presence of *L. casei* 4 hr prior to the addition of Toll-like receptor (TLR) agonists [poly(I:C) or LPS] with or without resveratrol. (b) IL-6 and IL-10 secretion in CSNs of DCs stimulated with single agents with or without resveratrol. The mean ± standard error of the mean (SEM) for five independent experiments is shown. Student's *t*-test (for the comparison of cytokine production with and without resveratrol) was performed: ***P* < 0.01; **P* < 0.05.



release was not regulated by resveratrol in any of these conditions. However, resveratrol reduced *B. animalis*- but not *L. casei*-induced IL-6 and IL-10 secretion in the absence of a TLR agonist (Fig. 5b), supporting the finding that resveratrol also inhibits TLR2 signalling.^{14,24}

We thus identified a unique and rather selective combination of one particular *Lactobacillus* strain (i.e. *L. casei* DN-114 001) and TLR3/TLR4 signals that acted in synergy to specifically enhance IL-12p70 production by DCs.

***Lactobacillus casei*/poly(I:C) synergy instructs DCs to boost Th1-polarized responses without inducing Th17**

Direct PAMP recognition by DCs with the ensuing TLR signalling is an obligatory condition for the development of a strong Th1 response to pathogens *in vivo*.²⁵ *In vitro*, co-operation between selected TLR agonists triggers a Th1 programme in human DCs.^{18,21} We thus hypothesized that *L. casei* plus poly(I:C), an *in vitro* model that mimics the context of viral intestinal infection, instructs DCs to boost Th1 polarization. DCs were exposed to *L. casei* 4 hr prior to the addition of poly(I:C), following which they were incubated for 40 hr, and then used to prime naïve CD4⁺ T cells. After 5–7 days, T cells were expanded in the presence of IL-2 and re-stimulated with PMA plus ionomycin. DCs stimulated with *L. casei* plus poly(I:C) induced higher amounts of IFN- γ than DCs exposed to *L. casei* or poly(I:C) alone (Fig. 6a). We found that T cells primed with DCs exposed to *L. casei* plus poly(I:C) showed an increased proportion of T-bet-positive cells among cells positive for high IFN- γ , providing additional evidence for enhanced Th1 development

(Fig. 6b). To investigate the role of IL-12 in the ability of DCs exposed to poly(I:C), *L. casei* alone or a combination of poly(I:C) and *L. casei* to increase IFN- γ production, we evaluated the effect of an antibody neutralizing IL-12. The presence of anti-IL-12 monoclonal antibody (mAb) during DC/T-cell co-cultures impaired Th1 differentiation, indicating that the IFN- γ production was critically dependent on the ability of DCs to secrete IL-12p70 (Fig. 6c). In a recent study, we found that DCs exposed to *L. casei* in the absence of a TLR agonist converted naïve T cells into suppressor T cells;¹⁶ in the present study, we found that T cells primed with *L. casei* plus poly(I:C)-treated DCs lost their capacity to exert an inhibitory function but T cells primed with *L. casei*-treated DCs did not (Fig. S3). These data provide evidence for two different and opposite effects of *L. casei* on DCs and demonstrate that *L. casei*-treated DCs require a TLR3 signal to become immunogenic.

Perturbation of homeostatic conditions by pathogen invasion drives the development of an immune response that must result in protection of the host without inducing inflammatory disorders. Th17 may be associated with the development of chronic inflammatory disorders^{26,27} as well as with host defence against selected bacterial infections of the gut.²⁸ Although DCs exposed to *L. casei* alone or together with TLR agonists released substantial amounts of Th17-polarizing cytokines that included IL-6, IL-1 β (Fig. S1a) and IL-23 (Fig. 7a), they were unable to drive a Th17-polarizing programme. Indeed, neither culture supernatants of *L. casei*/poly(I:C)-activated DCs nor the DCs themselves promoted Th17 polarization (Fig. 7b). As *L. casei* and poly(I:C) acted in synergy to

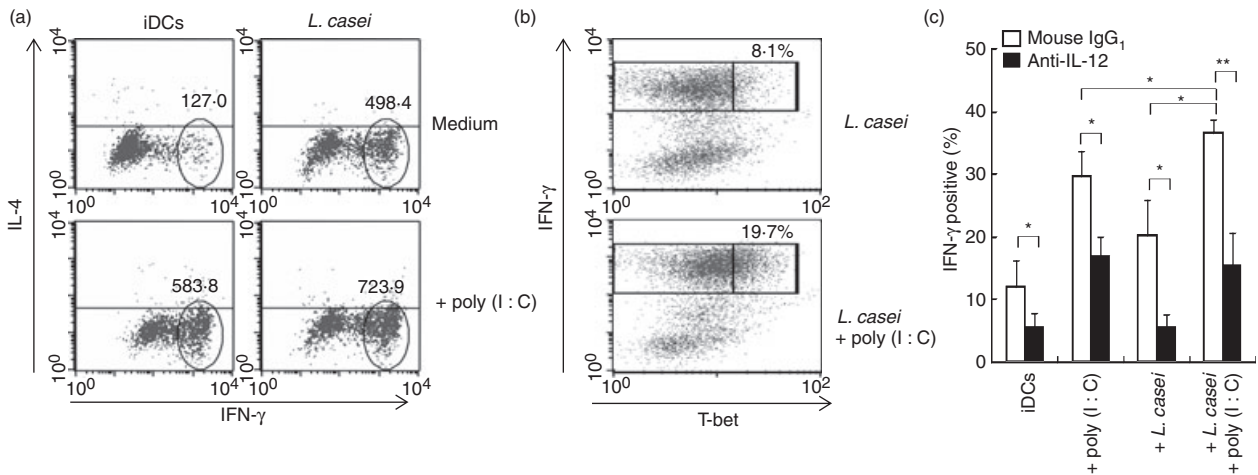


Figure 6. The combination of *Lactobacillus casei* and poly(I:C) instructs dendritic cells (DCs) to facilitate T helper type 1 (Th1) responses. DCs were stimulated with *L. casei* 4 hr prior to the addition of poly(I:C). Activated DCs were cultured for 5 days with allogeneic naïve CD4⁺ T cells and expanded with interleukin (IL)-2. After 3 days, primed T cells were re-stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin for 6 hr in the presence of monensin. (a) Interferon (IFN)- γ [mean fluorescence intensity (MFI)] and (b) the percentage of IFN- γ ⁺ T-bet⁺ cells were assessed using intracytoplasmic staining. One representative experiment of three is shown. (c) Neutralizing anti-IL-12 or isotype-matched control monoclonal antibody (mAb) was added during primary cultures. The percentage of IFN- γ ⁺ cells is shown as the mean \pm standard error of the mean (SEM) for five independent experiments. Student's *t*-test was performed: ***P* < 0.01; **P* < 0.05. iDC, immature dendritic cell.

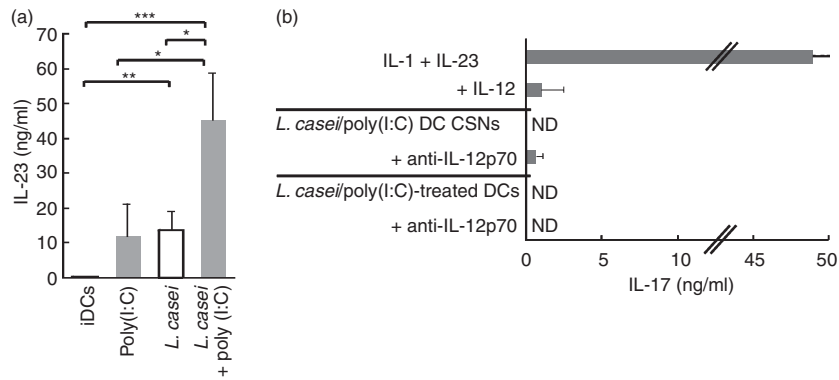


Figure 7. *Lactobacillus casei*/Toll-like receptor 3 (TLR3) signal co-operation does not promote T helper type 17 (Th17) responses. (a) Interleukin (IL)-23 secretion in the culture supernatants (CSNs) of dendritic cells (DCs) stimulated with *L. casei* and/or poly(I:C). The mean \pm standard error of the mean (SEM) for five experiments is shown. Student's *t*-test was performed: ****P* < 0.001; ***P* < 0.01; **P* < 0.05. (b) Plate-bound anti-CD3 activated naive T cells were cultured in the presence of anti-interferon (IFN)- γ , IL-23 and IL-1 with or without IL-12 [top: mean \pm standard deviation (SD) of two experiments] or the CSNs of *L. casei*/poly(I:C)-activated DCs with or without anti-IL-12p70 monoclonal antibody (mAb) (middle: mean \pm SD of four experiments). Purified naive T cells were co-cultured with *L. casei*/poly(I:C)-activated allogeneic DCs with or without anti-IL-12p70 mAb (bottom: mean \pm SD of four experiments). IL-17 production was measured by enzyme-linked immunosorbent assay (ELISA) after phorbol 12-myristate 13-acetate (PMA)/ionomycin re-stimulation. ND, not detectable.

induce IL-12p70 release, which inhibited IL-1- and IL-23-induced Th17 differentiation, we examined the impact of one mAb that selectively neutralized IL-12p70 but not IL-23 on Th17 development. IL-12p70 neutralization strongly inhibited IFN- γ production in T-cell primary cultures (Fig. S4). We found that anti-CD3-activated naive T cells stimulated in the presence of culture supernatants of *L. casei*/poly(I:C)-exposed DCs and anti-IL-12p70 mAb secreted small quantities of IL-17, which generally remained lower than those produced by IL-1- and IL-23-stimulated Th17 cells. Nevertheless, in spite of IL-12p70 neutralization at T-cell priming, *L. casei*/poly(I:C)-exposed allogeneic DCs consistently failed to induce Th17 differentiation.

We thus conclude that unique *L. casei*/TLR3 signalling on DCs induces large amounts of IL-12p70 and IL-23 which favour Th1 without driving a Th17 response.

Discussion

The mechanisms by which DCs translate information received from the microbial products they encounter are of significance in the maintenance of homeostasis and the establishment of protective immune responses.^{8,25} The intestinal mucosa is permanently exposed to trillions of potentially harmful bacteria, but the protective response of the host is selectively directed against pathogens.^{1,2} Following pathogen intrusion, the mechanisms by which DCs discriminate between virulent and non-virulent bacteria or viruses and then adapt their responses to elicit immunity versus tolerance remain to be elucidated.

Here we demonstrated that DCs exposed to at least one species of commensal-related bacterium, in this case

L. casei, can trigger a robust IL-12-dependent Th1 response, a process that is dictated by DC encounter with TLR3 agonists. However, naive T cells activated in the presence of cell-free supernatants of *L. casei* combined with a TLR3 agonist or DCs themselves were not polarized towards Th17 in spite of the release by DCs of substantial amounts of IL-6, IL-1 β and IL-23, and neutralization of IL-12p70 in T-cell primary cultures. This may reflect inappropriate DC signalling and/or the inability of monocyte-derived DCs to direct Th cell fate towards Th17.¹² In this regard, a recent study reported that the supernatant of yeast zymozan-activated monocyte-derived DCs induced IL-17 secretion by human CD45RO⁻ T cells.²⁹ It has been clearly demonstrated that commensal bacteria interact with the TLR expressed by gut epithelial cells to indirectly control DC function and dendrite extension.^{13,30} Nonetheless, a direct non-invasive bacterium/DC interaction can be visualized *in vivo*³⁰ and the importance of DC-associated antigen in the induction of oral tolerance or a protective immune response is well documented.³¹ Our data indicate that exposure to poly(I:C) after, at the same time as, or prior to exposure to *L. casei* had a synergistic effect on DCs, selectively inducing IL-12p70 secretion. The latter was down-regulated by the addition of resveratrol, suggesting the involvement of TRIF-coupled receptor signalling pathways. We then postulated that this synergy was not restricted to *L. casei* and the TLR3 agonist. We found that *L. casei* moderately facilitated LPS-induced IL-12p70 production. In contrast to poly(I:C), when LPS was added together with or prior to *L. casei*, it inhibited IL-12p70 production. As the gut is constantly loaded with large amounts of LPS, DCs may be refractory to CB-induced IL-12p70 release, unable to induce a Th1

programme and thus poised to maintain gut tolerance. However, direct contact between DCs and CB and viruses may be occurring *in vivo*. Because DC exposure to *L. casei* and poly(I:C) boosted IL-12-driven Th1 responses, we propose acute viral intestinal disease as a relevant *in vivo* context for our *in vitro* observations. This proposal is based on the consensus that probiotics contribute significantly to the relief of acute diarrhoea caused by rotavirus [a double-stranded RNA (dsRNA) virus] but not to that caused by coronavirus (an ssRNA virus).³² *Lactobacillus casei* in fermented milk attenuates the incidence and duration of diarrhoea in infants.³³ Similarly, elderly patients who regularly consumed a probiotic drink containing *L. casei* displayed a reduced incidence of diarrhoea.³⁴ Finally, *L. casei* administered in food protects rats from rotavirus-associated diarrhoea.³⁵ The manipulation of the dominant microbial community by probiotics is a prerequisite for the exertion of such potentially beneficial effects. In this regard, recent reports have demonstrated the existence of functional interactions between the common Gram-negative commensal-related bacterium *B. thetaiotaomicron* and a selective *Bifidobacterium* strain that result in changes in the bacterial and host genes.^{36,37}

The importance of TLR3/TLR4 signalling *in vivo* is further illustrated by the control of viral infection in IL-1R-associated kinase-4 (IRAK)-4 deficient patients lacking TLR7/TLR8/TLR9-dependent pathways.³⁸ Because two TLR signals (one TRIF-coupled TLR signal plus one endosomal receptor signal) must be delivered to human monocyte-derived DCs to sustain their IL-12 production and induce strong Th1-polarizing capacity,^{18,21} here we propose that *L. casei* may substitute for one signal to facilitate IL-12p70 and Th1 protective responses. Signalling pathways through MyD88 and interferon regulatory factor 5 (IRF)5 are required for IL-12p40 and IL-23p19 gene activation, respectively, whereas TRIF/IRF3 transactivates and amplifies IL-12p35.^{39,40} Here we found that *L. casei*/poly(I:C) co-operation enhanced IL-12p70 and IL-23 secretion. However, the molecular pathways involved in *L. casei*/TLR3 synergy remain to be elucidated. Taken together, our results show that DCs may integrate signals received from commensal flora and viral products, extending the concept that selected TLR agonist combinations synergistically trigger IL-12 production by DCs and the Th1-polarizing programme.^{18,21} Therefore, the search for additional critical CB/TLR combinatorial codes and elucidation of the molecular pathways involved may open avenues for the use of probiotics as potential adjuvants for the delivery of efficient mucosal vaccines.

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Author contributions

NB performed research, analysed data, and wrote the paper; MR performed research; RB-S and SS contributed vital new reagents; MS designed the research, analysed data and wrote the paper.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Cytokine profile of dendritic cells (DCs) stimulated with *Lactobacillus casei* or *Bifidobacterium animalis* in combination with various Toll-like receptor (TLR) agonists.

Figure S2. Cytokine profile of dendritic cells (DCs) stimulated with poly(I:C) or lipopolysaccharide (LPS) in combination with various strains of commensal-related bacteria (CB).

Figure S3. *Lactobacillus casei*/Toll-like receptor 3 (TLR3)-treated dendritic cells (DCs) lose their capacity to induce suppressive T cells.

Figure S4. Anti-interleukin (IL)-12p70 strongly inhibits interferon (IFN)- γ production in T-cell primary culture.

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