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Innate inflammatory responses to the Gram-positive bacterium *Lactococcus lactis*

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Summary *Lactococcus lactis* is a non-pathogenic and non-colonizing Gram-positive bacterium commonly used in the dairy industry. To support the potential applications of this bacterium, such as use as an oral live vaccine, it is of interest to investigate the adjuvant properties of *L. lactis*. We compared the proinflammatory effects of *L. lactis* with two non-pathogenic Gram-negative bacteria: *Escherichia coli* and *Salmonella typhi*, a widely studied live vaccine. The gene expression profiles of chemokines induced by the three bacteria were examined in macrophages *in vitro* and in cells recruited into murine air-pouches *in vivo*. In addition, we studied the effect of co-incubating bacteria with dendritic cells (DCs) generated from mice bone marrow. We demonstrate that *L. lactis* exhibits proinflammatory effects, which indicates a capacity for adjuvanticity by this bacterium.

Introduction

Lactococcus lactis is a Gram-positive lactic acid bacterium (LAB) that is commonly used in the diary industry and is readily found in foods. *L. lactis* is non-pathogenic and non-colonizing, and has been given Generally Recognized As Safe (GRAS) status by the U.S. Food and Drug Administration [1]. An application of *L. lactis* is its use as a live bacterial vector to deliver biological agents [2]. For example, *L. lactis* was genetically modified to heterologously express recombinant vaccine antigens such as fragment C of the tetanus toxin [3] and the E7 antigen of human papilloma virus (HPV) [4]. These live strains of *L. lactis* were able to elicit specific immune responses against the antigens in vaccinated mice. *L. lactis* was also used to express and deliver cytokines such as interleukins (IL)-2, -6, -10 and -12 in mice [5–7]. A phase I clinical trial using *L. lactis* expressing human IL-10 as a therapeutic treatment for inflammatory bowel disease was completed in 2006 and demonstrated that mucosal drug delivery using *L. lactis* was effective and clinically well-tolerated [8]. The use of *L. lactis* as a live bacterial delivery system is highly promising. Although there are many current studies on the

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utilization of genetically modified *L. lactis*, little is known regarding the specific interactions of this bacterium with the immune system. To support the potential uses of this bacterium, it is of interest to examine the adjuvant properties of *L. lactis*.

Other bacteria are also being investigated for use as live recombinant vaccines [9]. These include non-pathogenic ''harmless'' bacteria that are found in foods and commensal bacteria that are part of the human microflora. Conversely, attenuated pathogenic bacteria may also be used to generate live recombinant vaccines. For instance, non-pathogenic strains of *Salmonella* are a well-studied example [10]. Successes in animal studies with recombinant *Salmonella* have led to human trials of live vaccines against tetanus [11] and HIV [12]. Heterologous antigen delivery using an attenuated strain of a rabbit enteropathogenic *Escherichia coli* is also under investigation [13].

Chemokines play an important role in many biological functions, such as innate and adaptive immunity, wound healing, metastasis and angiogenesis [14]. Some chemokines are involved in the innate immune response by recruiting effector leukocytes. Following assault by a pathogen, conserved pathogenic patterns such as lipopolysaccharides (LPS) of Gram-negative bacteria or peptidoglycan (PGN) of Gram-positive bacteria can activate pattern-recognition receptors (PPRs) on phagocytes. For example, LPS activation of Toll-like receptors (TLR) or PGN activation of NOD-like receptors on epithelial and antigen presenting cells (APCs) leads to the production of various cytokines and inducible chemokines, which function to direct the immune response [15]. Assorted chemokines will selectively recruit neutrophils, monocytes/macrophages, dendritic cells (DCs) and natural killer (NK) cells to the site of inflammation. The specific chemokines produced will contribute to the type of T cell response produced, T helper 1 (Th1) or T helper 2 (Th2) [16]. Therefore, chemokines are important mediators that instruct the adaptive immune response.

DCs are APCs that function to link the innate and adaptive immune responses. These cells express PPRs and are able to specifically respond to different microbial stimuli [17]. Once stimulated, immature DCs undergo maturation and eventually contribute to the activation of T cells for an adaptive immune response. These two tasks of DCs connect the innate and adaptive immune responses: detection and acquisition of antigens, and the activation of lymphocytes. Furthermore, chemokines and cytokines produced by APCs including DCs are also involved in the induction, amplification, and direction of T cell responses [18].

In this study, we compared the proinflammatory effects of *L. lactis* strain NZ9000, a non-pathogenic bacterium, with *E. coli* strain DH5α and *Salmonella typhi* strain Ty21a, both non-pathogenic strains of pathogenic bacteria [19,20]. Significantly, we compare *L. lactis* with a well-studied bacterial live vaccine vector, *S. typhi* Ty21a. We show that *L. lactis* exhibits proinflammatory effects, which is an important adjuvant property. *L. lactis* induced chemokine mRNA expression both *in vitro* and *in vivo*, but to different levels than stimulation with *E. coli* and *S. typhi*. However, all bacteria stimulated the recruitment of leukocytes *in vivo* and the maturation of DCs *in vitro* to similar levels. These results demonstrate proinflammatory effects of *L. lactis*, therefore permitting a further understanding of its adjuvant properties.

Materials and methods

Bacterial strains and growth conditions

L. lactis subsp. cremoris strain NZ9000 (herein referred to as *L. lactis*) was grown without shaking in M17 medium (Oxoid; Basingstoke, Hampshire, England) supplemented with 0.5% glucose (GM17) at 30 °C. This strain of *L. lactis* is a plamid-free derivative of the dairy starter strain NCDO71 that is suitable for use as a live vaccine vector [21]. *E. coli* strain DH5α (herein referred to as *E. coli*) was grown with shaking in LB broth at 37 °C. The non-pathogenic vaccine strain of *S. typhi* Ty21a (herein referred to as *S. typhi*) [20] was obtained from the Salmonella Genetic Stock Center (Calgary, AB, Canada) and was grown with shaking in LB broth at 37 °C. In all experiments, bacteria were freshly diluted from saturated overnight cultures at 1/100 for *L. lactis* and *E. coli* or 6/100 for *S. typhi* and grown until late log phase was reached at OD_{600} = 1.5—1.7. Then bacteria were washed in PBS and resuspended to the desired concentration in cell culture medium or PBS. Serial dilutions and plating on GM17 or LB agar plates were performed to count colony-forming units (CFU) and confirm correct bacterial concentrations.

Cell culture

The B10R macrophage cell line [22] was maintained in DMEM medium (Gibco; Burlington, ON, Canada) supplemented with 10% FBS (Gibco), 0.3 mg/ml L-glutamine, 1 mM penicillin—streptomycin and 0.5 mM β-ME. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. For co-incubation assays, $\sim 10^6$ cells were grown in a T25 flask (4 ml), and then stimulated for 4 h with different concentrations of bacterial cells resuspended in DMEM. As negative or positive controls, macrophages were left unstimulated (nil) or stimulated with 100 ng/ml LPS, respectively. Data represent three individual co-incubation experiments.

Ribonuclease protection assay (RPA)

Following stimulation, bacteria were washed off with PBS and adherent macrophages were harvested in 1 ml TRIzol reagent (Invitrogen; Burlington, ON, Canada) for total RNA extractions as described in the manufacturer's protocol. Levels of chemokine mRNA was assessed using the BD RiboQuant Ribonuclease Protection Assay (RPA) kit with the mCK-5c (mouse chemokine) probe set (BD Biosciences; Mississauga, ON, Canada) as we previously performed [23]. This method allows the concurrent evaluation of the mRNA levels of several chemokines and controls: CCL1/TCA-3, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CXCL2/MIP-2, CXCL10/IP-10, and house-keeping genes L32 and GAPDH. Briefly, 10 μg of total RNA was hybridized overnight with the [α-^{32}P]UTP radiolabeled RNA probe set. Following RNase and proteinase K treatments, the remaining protected RNA probes were isolated by phenol/chloroform extraction, precipitated with ethanol and then resolved on

denaturing polyacrylamide gel. Finally, the gel was dried and bands were detected on autoradiographic film and by phosphorimaging. Quantification was performed using the QuantityOne software from BioRad (Mississauga, ON, Canada). Levels of chemokine mRNA was normalized to mRNA levels of GAPDH, and then taken as the fold increase from unstimulated (nil) negative controls.

Murine air-pouch assay

Sterile air-pouches were created on the dorsal region of 6 to 8-week-old male BALB/c mice (Charles River Laboratories; St. Constant, QC, Canada) and air-pouch exudates were assessed as previously described [24,25]. Briefly, 3 ml of sterile air was injected subcutaneously on days 1 and 3 to create the air-pouch, and then the stimulus was injected into the pouch on day 7. Bacterial cultures (10^8 CFU) were resuspended in endotoxin-free PBS, positive and negative controls consisted of mice inoculated with 10 μg LPS or PBS only, respectively. Air-pouch exudates were recuperated 6 h after inoculation. Total leukocytes in air-pouch exudates were counted on a hemocytometer using Trypan blue exclusion staining. Differential cell counts were performed microscopically on Cytospin slides stained with DiffQuik. Finally, total leukocytes were lysed in TRIzol reagent (Invitrogen) for RNA extraction and chemokine mRNA analysis by RPA.

All procedures were in accordance with guidelines of the Canadian Council on Animal Care, as approved by the Animal Care Committee of McGill University.

Isolation of murine bone marrow derived dendritic cells and flow cytometry

Dendritic cells (DCs) were cultured from mice bone marrow essentially as previously described [26,27]. Bone marrow cells (1×10^6) from 8 to 10-week-old male BALB/c mice were plated in a total of 4 ml RPMI (Wisent; St. Bruno, QC, Canada) in 6-well plates supplemented with 10% FBS (Wisent), 0.3 mg/ml L-glutamine, 1 mM penicillin—streptomycin and 0.5 mM β-ME. Mouse recombinant GM-CSF (Biosource; Camarillo, CA, USA) was added on day 0 to a final concentration of 250 U/ml. On day 2, half of the media was removed and replaced with fresh RPMI supplemented with GM-CSF to a final concentration of 500 U/ml. Cells were washed and fed again on day 3 and 6 maintaining a GM-CSF concentration of 500 U/ml. Dendritic cells were stimulated overnight on day 6 with 10^7 bacteria/well, 1 μg/ml LPS (positive control), or left unstimulated (negative control). Dendritic cells were stimulated directly in the 6-well plates to reduce manipulation. The percentage of viable dendritic cells recuperated following differentiation and stimulation was at 40—50% for all groups. Antibiotics in the cell culture media were sufficient to prevent bacterial growth during the overnight co-incubation (data not shown).

Following stimulation, DCs were stained for flow cytometric analysis or cell sorting as previously described [28]. Cell were washed in PBS containing 2% FBS and 0.1% NaN_3, then non-specific Ab binding was prevented by incubating cells in 2.4G2 mAb for 25 min on ice. All Ab incubations were performed on ice in the dark for 25 min. Unless otherwise indicated, all Abs were purchased from BD Biosciences. Cells were triple stained for flow cytometric analysis or singly stained for cell sorting.

Cells were triple stained with anti-CD11c, anti-mouse-I-A^b (MHC class II; MHC-II) and anti-CD86 Abs (Cederlane Laboratories; Burlington, NC, USA) for flow cytometric analysis. Ab incubations and washes with PBS containing FBS and NaN_3, as described above, were performed in the following sequence: biotin-conjugated anti-CD11c, wash, FITC-conjugated streptavidin (SA), wash twice, biotin-conjugated anti-MHC-II, wash, Cy5-conjugated SA and PE-conjugated CD86. Cells were resuspended in PBS and analyzed with the FACScalibur system using CellQuestPro software (BD Biosciences).

Cells stained with PE conjugated anti-CD11c Ab (StemCell Technologies; Vancouver, BC, Canada) were collected with the FACSAria system using FACSDiva software (BD Biosciences). Purity of CD11c+ cells were always at above 98%.

cDNA synthesis and qPCR

Total RNA from CD11c+ sorted DCs was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was prepared using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen) on 3 μg RNA with oligo(dT) primers. Levels of cytokine mRNA was determined by quantitative PCR (qPCR) using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) with 1 μl of cDNA. qPCR was performed and analyzed with RotorGene 3000 real-time PCR analyzer (Corbett Life Sciences; Sydney, Australia). Pairs of primers used are as follows: GAPDH, 5′-CACTCACGGCAAATTCAACGGC-3′, 5′-TAGTGTTTGTACCCCCGTAGCC-3′; IL-1β, 5′-CAACCAACAAGTGATATTCTCCATG-3′, 5′-GATCCACACTCTCCAGCTGCA-3′; IL-12p40, 5′-GGAAGCACGGCAGCAGAATA-3′, 5′-AACTTGAGGGAGAAGTAGGAATGGGGA-3′; IL-10, 5′-TTCAGCCAGGTGAAGACTTTC-3′, 5′-TGGGGCATCACTTCTACCAG-3′.

Statistical analysis

Statistical significance between groups was determined using the ANOVA function of the StatView program, version 5.0 (SAS Institute Inc.). $p < 0.05$ was considered statistically significant.

Results

L. lactis induces chemokine expression at lower levels than *E. coli* in B10R macrophages *in vitro*

Although already used as a live vaccine vector [3,4,29—32], few studies have examined the immune activation and adjuvant properties of *L. lactis*. As macrophages are the primary cell type to detect invading microorganisms contributing to the induction of innate inflammatory and microbiocidal responses, we first examined the effect of *L. lactis* on a macrophage cell line *in vitro*. Increasing concentrations of *L. lactis* bacteria (10^4—10^9) were co-incubated with B10R murine macrophages (~10^6 cells) for 4 h. Bacterial cells were washed and the macrophages were harvested to exam-

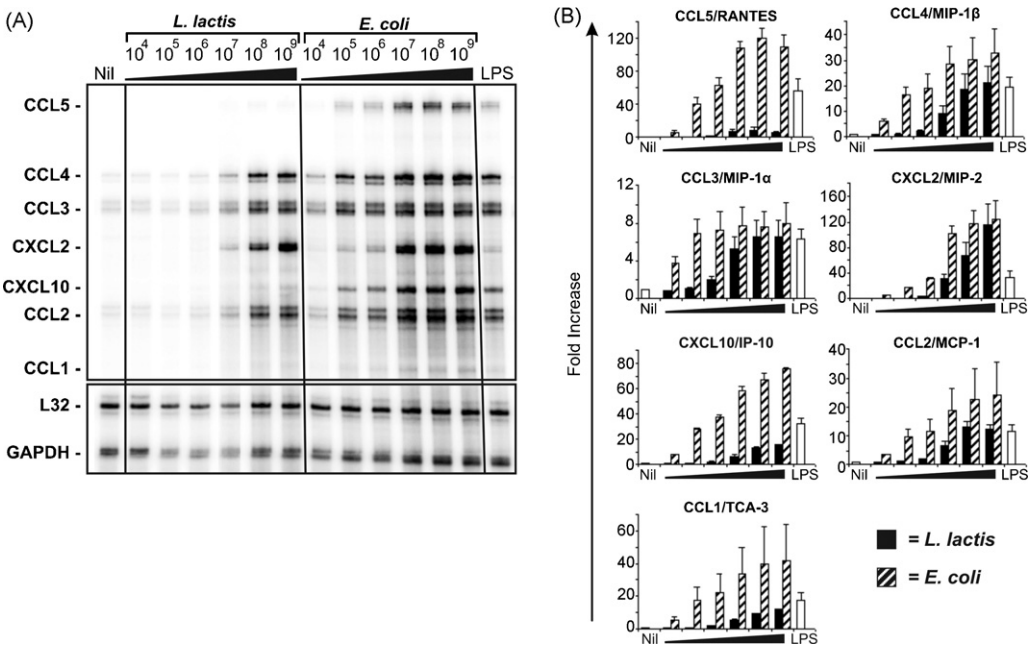

Figure 1 *L. lactis* and *E. coli* dose response of B10R macrophage chemokine induction *in vitro*. 10^4 to 10^9 CFU of *L. lactis* or *E. coli* were co-incubated with 10^6 B10R macrophages for 4 h. Macrophages stimulated with 100 ng/ml LPS or left unstimulated (nil) are presented as positive and negative controls, respectively. (A) Levels of chemokine mRNA were measured by RPA, a representative gel is shown. (B) Densitometric quantification of chemokine mRNA levels were normalized to levels of GAPDH mRNA and presented as the fold increase from unstimulated (nil) negative control. Data presented are the means and standard errors of three individual experiments.

ine mRNA levels of various chemokines by Ribonuclease Protection Assay (RPA).

Macrophage stimulation with *L. lactis* was capable of inducing transcription of all but one chemokine tested (Fig. 1). Remarkably, the mRNA levels of the CCL5/RANTES chemokine remained near basal levels even following stimulation at the maximal dose of *L. lactis*, 10^9 colony forming units (CFU). Significant levels ($p < 0.05$ to nil) of the remaining chemokines were reached at *L. lactis* stimulation doses of 10^7 (CCL3/MIP-1α, CCL2/MCP-1, CCL1/TCA-3) or 10^8 CFU (CCL4/MIP-1β, CXCL2/MIP-2, CXCL10/IP-10). These results show that this non-pathogenic bacterium is indeed recognized by B10R macrophages and is able to induce expression of various chemokines *in vitro*. This represents a direct illustration of a proinflammatory effect of *L. lactis*.

Next, we compared the induction of chemokine expression in B10R macrophages by *L. lactis* with a non-pathogenic strain of *E. coli*. This bacterium was stronger at stimulating chemokine mRNA expression in macrophages than *L. lactis*. Significant levels ($p < 0.05$ to nil) of CCL5/RANTES mRNA was reached at a dose of 10^5 CFU of *E. coli*, while all doses of *L. lactis* failed to induce this chemokine (Fig. 1). Similarly, *E. coli* and *L. lactis* induced highly differing levels of the CXCL10/IP-10 chemokine.

Other chemokines were induced to significant levels ($p < 0.05$ to nil) by *E. coli* at lower stimulation doses than *L. lactis*. To induce CCL3/MIP-1α and CXCL2/MIP-2 gene expression, 10^5 CFU of *E. coli* was equivalent to 10^7 CFU of *L. lactis*, and 10^7 CFU of *E. coli* was equivalent to 10^8 CFU of *L. lactis*, respectively (Fig. 1). The remaining chemokines (CCL4/MIP-1β, CCL2/MCP-1, CCL1/TCA-3) were induced to similar levels by *E. coli* and *L. lactis*, although the trend persists that *E. coli* is more inflammatory than *L. lactis* (Fig. 1).

Both *L. lactis* and *E. coli* exhibit proinflammatory properties as they are capable of inducing production of chemokine mRNA from B10R macrophages. However, our results show that *L. lactis* may initiate a more moderate inflammatory response than *E. coli*.

L. lactis induces chemokine expression at lower levels than *S. typhi* in B10R macrophages *in vitro*

We next compared the effects of *L. lactis* and *E. coli* on B10R macrophages with a commonly used live vaccine strain of *S. typhi* [20]. A dose response experiment assessing the effect of increasing concentrations of *S. typhi* on B10R macrophages was performed (data not shown). *S. typhi* was found to exhibit a similar induction profile of chemokines as *E. coli*. To compare the chemokine induction profile of all three bacterial strains concurrently, we chose a dose of 10^7 CFU. At this dose, both *E. coli* and *S. typhi* significantly stimulated ($p < 0.05$ to nil) all chemokines, while *L. lactis* failed to stimulate some chemokines (CCL5/RANTES, CXCL10/IP-10, and CCL1/TCA-3) and stimulated the remainder to lower levels (Fig. 2). The chemokines CCL4/MIP-1β and CCL2/MCP-1 were induced to similar levels by *E. coli* and *S. typhi* but 3-fold higher than by *L. lactis* (Fig. 2B). With CCL5/RANTES and CXCL2/MIP-2, *S. typhi* stimulated these chemokines to intermediate levels between *E. coli* (highest) and *L. lactis* (lowest) (Fig. 2B). Finally, all three bacte-

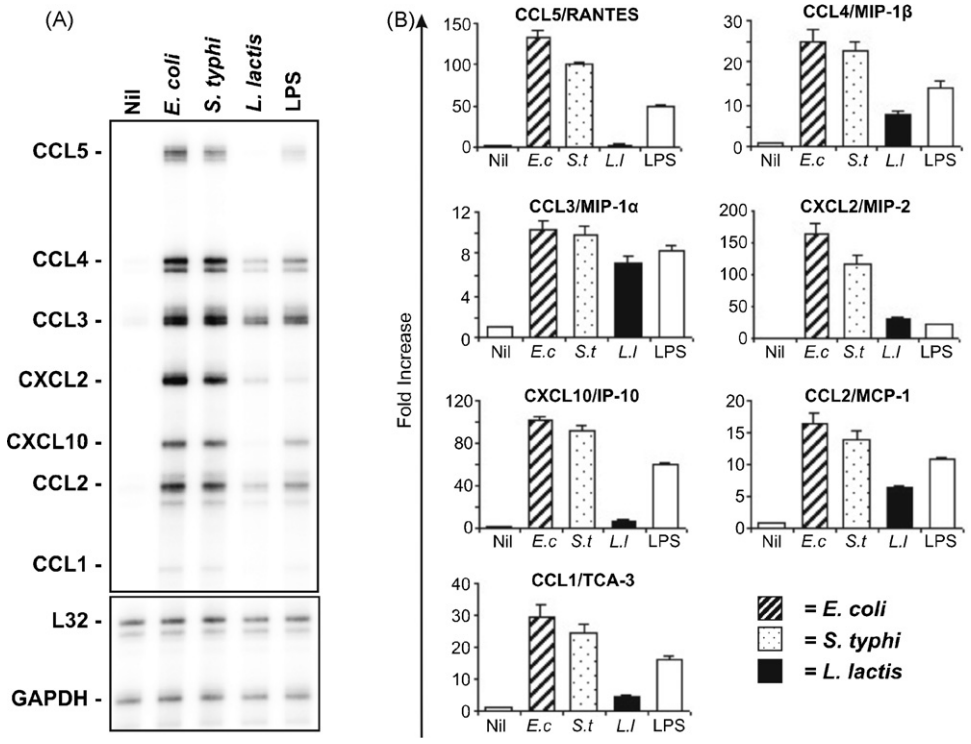

Figure 2 Induction of chemokine expression in B10R macrophages by *L. lactis*, *E. coli* and *S. typhi in vitro*. 10^7 CFU of *L. lactis*, *E. coli* or *S. typhi* were co-incubated with 10^6 B10R macrophages for 4 h. Macrophages stimulated with 100 ng/ml LPS or left unstimulated (nil) are presented as positive and negative controls, respectively. (A) Levels of chemokine mRNA were measured by RPA, a representative gel is shown. (B) Densitometric quantification of chemokine mRNA levels were normalized to levels of GAPDH mRNA and presented as the fold increase from unstimulated (nil) negative control. Data presented are the means and standard errors of three individual experiments.

ria stimulated expression of the CCL3/MIP-1α chemokine to similar levels.

Taken together, these co-incubation assays with B10R macrophages illustrate a proinflammatory effect of *L. lactis*, which was shown capable of inducing expression of chemokine mRNAs *in vitro*. However, *L. lactis* was found to show a significantly lower chemokine expression profile than *E. coli* and *S. typhi*.

Recruitment of leukocytes by *L. lactis in vivo*

After assessing the *in vitro* effects of *L. lactis*, *E. coli* and *S. typhi*, the next experiments sought to compare their *in vivo* proinflammatory effects. We performed murine air-pouch assays where we first generated a sterile, epithelium enclosed, air-pouch on the dorsal region of a mouse [24,25]. Although *L. lactis* interacts with the epithelia of mucosal sites following oral delivery, the air pouch assay allows the recuperation and assessment of recruited cells. The recruitment of immune cells was stimulated by inoculating each air-pouch with either 10^8 CFU of bacteria, 10 μg LPS as a positive control or PBS as a negative control (nil). Epithelial cells lining the air-pouch and resident phagocytes reacted to the stimuli, and caused the recruitment of immune cells into the pouch. Six hours following the injection of bacteria, contents of the air-pouches were recuperated and the recruited cells were characterized.

Firstly, we observed that the presence of any bacteria (*L. lactis*, *E. coli* or *S. typhi*) in a murine air-pouch was capable of inducing significant ($p < 0.05$ to nil) recruitment of leukocytes (Fig. 3). Stimulation with each bacterium resulted in comparable levels of cellular recruitment; ranging from 8 to 11×10^5 leukocytes (Fig. 3). This result indicates that, *in vivo*, *L. lactis* exhibits equivalent potency to stimulate cellular recruitment as *E. coli* and *S. typhi*, the latter being the more widely used bacteria as a live vaccine.

Cytospin slides were prepared of the air-pouch exudates and were used to carry out differential cell counts by microscopy. Of all the leukocytes recruited by *L. lactis*, *E. coli* and *S. typhi*, the great majority were neutrophils (Table 1). With the bacterial groups and the LPS positive control, the percentage of recruited leukocytes found to be neutrophils varied from 88% to 95% (Table 1). The proportion of neutrophils, eosinophils, lymphocytes and monocytes/macrophages recruited by the bacteria were not significantly different when compared to each other or the LPS positive control.

In vivo stimulation of chemokine expression in recruited leukocytes by *L. lactis*

We investigated the general activation status of the leukocytes recruited within the air-pouches by determining the

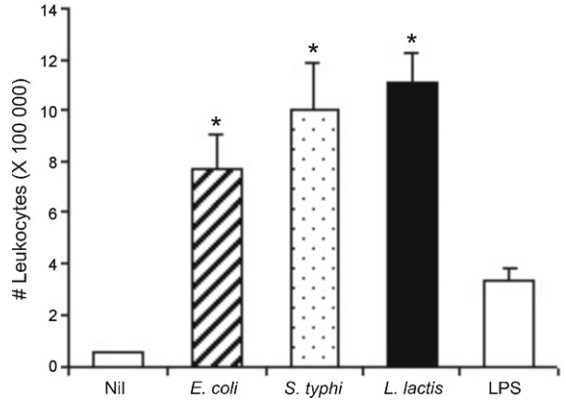

Figure 3 Relative leukocyte recruitment by *L. lactis*, *E. coli* and *S. typhi* into murine air-pouches. Air-pouches were prepared on the backs of BALB/c mice and inoculated with 10^8 CFU of *L. lactis*, *E. coli* or *S. typhi*, or as positive and negative controls, inoculated with 10 μg LPS or PBS, respectively. Air-pouch exudates were recuperated 6 h after inoculation. Total number of recruited leukocytes in exudates was determined by counting on a hemocytometer using Trypan blue exclusion staining. Data presented are the means and standard errors of four mice per group. $^*p < 0.05$ to nil.

mRNA levels of various chemokines by RPA. In comparison to the effect of *L. lactis*, *E. coli* and *S. typhi* on B10R macrophages *in vitro*, a noticeably different chemokine profile was observed with the recruited leukocytes, which were mainly neutrophils.

With B10R macrophages, *E. coli* and *S. typhi* highly stimulated expression of CCL5/RANTES mRNA. However, in recruited leukocytes *in vivo*, neither *L. lactis*, *E. coli* nor *S. typhi* stimulated detectable levels of the CCL5/RANTES chemokine (Fig. 4). On the other hand, all the bacterial species stimulated high levels of CCL4/MIP-1β, CCL3/MIP-1α and CXCL2/MIP-2 (Fig. 4). *L. lactis* stimulated higher levels of CCL1/TCA-3 and CCL2/MCP-1, while *S. typhi* stimulated very high levels of CXCL10/IP-10 in comparison to the other stimuli. Therefore, although *L. lactis*, *E. coli* and *S. typhi* can stimulate similar levels of leukocyte recruitment into murine air-pouches, these recruited cells display an exclusive activation status according to the bacterial stimuli. These results show that *L. lactis* is capable of inducing chemokine expression both *in vitro* and *in vivo*, and displays comparable potency as *S. typhi*, an established live vaccine.

L. lactis stimulates maturation of murine bone marrow derived dendritic cells *in vitro*

A function of the innate immune response is to detect the presence of a pathogenic attack. Macrophages and neutrophils are usually the first cells to phagocytose and clear bacterial infections. However, DCs are considered the more efficient antigen presenting cell (APC) of bacterial epitopes, which will function to direct the adaptive immune response accordingly. DCs are known to undergo maturation upon interaction with bacteria [33,34].

Myeloid DCs were grown *in vitro* from mice bone marrow, and then co-incubated with 10^7 CFU of *L. lactis*, *E. coli* and *S. typhi*, or with LPS (1 μg/ml) as a positive control. Following 24 h of stimulation, non-adherent proliferating DCs were harvested, washed and analyzed by flow cytometry. Viable DCs were gated using the CD11c marker, which were then analyzed for levels of MHC-II and the CD86 costimulatory molecule (Fig. 5A). While all DCs express the CD11c marker, immature DCs express low levels of both MHC-II and CD86, but maturation results in high expression of both these cell-surface molecules.

Unstimulated (nil) negative controls displayed a basal level of mature DCs, double positive for MHC-II and CD86, which was at about 10% (Fig. 5B). Stimulation with LPS or bacteria caused a statistically significant ($p < 0.05$ to nil) increase in the percentage of mature DCs (Fig. 5B). However, no statistical difference in the percentage of mature DCs was observed among the groups.

Induction of cytokine expression in murine dendritic cells by *L. lactis*

The role of mature DCs once they migrate to regional lymph nodes is to stimulate activation of T cells, thereby inducing an adaptive immune response. DCs also function to direct the type of immune response, Th1 or Th2, by specifically secreting cytokines and chemokines [18]. Therefore, we examined mRNA expression of cytokines by DCs stimulated to mature by bacteria. Following co-incubation with bacteria, CD11c+ DCs were collected by FACS, and then levels

Table 1 Leukocyte recruitment by *L. lactis*, *E. coli* and *S. typhi* into murine air-pouches differentiated by cell type

Group	Cell type[a]				Total leukocytes ×100,000
	Neutrophils ×10,000	Eosinophils ×1,000	Lymphocytes ×100	Monocytes/macrophages ×1,000	
Nil	2.57 (45.5% ± 5.9)	8.06 (14.3% ± 4.1)	23.27 (4.1% ± 1.3)	20.26 (35.9% ± 3.7)	0.56
E. coli	68.56 (89.2% ± 1.6)	75.89 (9.9% ± 1.3)	28.82 (0.4% ± 0.2)	4.16 (0.5% ± 0.2)	7.69
S. typhi	88.39 (88.3% ± 1.7)	112.16 (11.2% ± 1.6)	4.17 (0.0% ± 0.0)	4.17 (0.4% ± 0.2)	10.01
L. lactis	105.52 (95.0% ± 0.5)	41.65 (3.8% ± 0.5)	13.88 (0.1% ± 0.1)	12.50 (1.1% ± 0.2)	11.11
LPS	31.53 (92.7% ± 0.7)	19.09 (5.6% ± 1.0)	5.67 (0.2% ± 0.1)	5.29 (1.6% ± 0.5)	3.40

[a] Values are average counts for each cell type. Values in brackets are the average and standard error of the proportion of each cell type. Data represent four mice per group.

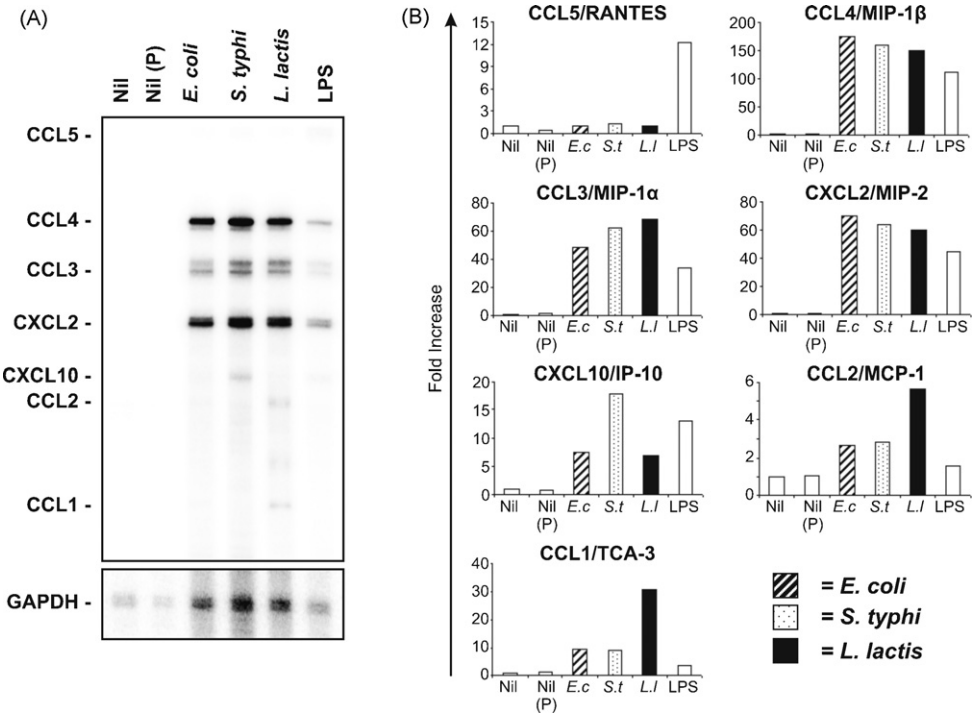

Figure 4 Induction of chemokine expression in leukocytes recruited into murine air-pouches by *L. lactis*, *E. coli* and *S. typhi*. Air-pouches were prepared on the backs of BALB/c mice and inoculated with 10^8 CFU of *L. lactis*, *E. coli* or *S. typhi*, or as positive and negative controls, inoculated with 10 μg LPS or PBS, respectively. Air-pouch exudates containing recruited leukocytes were recuperated 6 h after inoculation. (A) Levels of chemokine mRNA were measured by RPA, a representative gel is shown. (B) Densitometric quantification of chemokine mRNA levels were normalized to levels of GAPDH mRNA and presented as the fold increase from PBS negative control (nil). Data represent four mice per group. Nil, PBS negative control; nil (P), air-pouch lining of PBS negative control.

of cytokine mRNA was quantified by reverse-transcriptase qPCR.

IL-1β is a proinflammatory cytokine that was demonstrated to be an effective mucosal adjuvant [35]. The function of IL-1β as an adjuvant is suggested to be derived from the induction of other cytokines, which create a microenvironment suitable for efficient systemic and mucosal immune responses [35]. *L. lactis* was capable of inducing significant levels of IL-1β mRNA from DCs; resembling DC stimulation with *E. coli* and *S. typhi* ($p < 0.05$ to nil) (Fig. 6). Another proinflammatory cytokine is IL-12, which functions to induce Th1 responses and is used as an effective adjuvant to promote cell-mediated immunity [36]. Expression of IL-12 is primarily regulated by transcription of its p40 subunit [37]. In bacteria-stimulated DCs, the level of IL-12p40 mRNA was most elevated by stimulation with *L. lactis* ($p < 0.01$ to nil) (Fig. 6). To a lesser extent, *S. typhi* was also capable of stimulating statistically significant ($p < 0.05$ to nil) levels of IL-12p40 mRNA. Therefore, the induction of IL-1β and IL-12 by *L. lactis* suggests that this bacterium may exhibit adjuvant properties.

Surprisingly, *L. lactis* also very strongly induced mRNA levels of IL-10 ($p < 0.0001$ to nil) (Fig. 6). This cytokine is considered to exhibit dual functions: as an activator of humoral immune responses by stimulating B cells and as a down-regulator of the immune system [38]. Induction of IL-10 by *L. lactis* may suggest the activation of humoral immune responses.

Discussion

Given the recent concerns on probiotic treatments involving mixes of bacteria [39], it is important to individually investigate strains of immune stimulating bacteria. In this study, we show direct evidence of the proinflammatory effects of *L. lactis*, a promising live vaccine vector. Co-incubation of *L. lactis*, *E. coli* or *S. typhi* bacteria with B10R macrophages *in vitro* was able to stimulate the transcription of various chemokines. Interestingly, *L. lactis* stimulated most chemokines to lower levels than *E. coli* and *S. typhi*, which are both Gram-negative bacteria. This correlates with previous studies that showed Gram-negative bacteria such as *Klebsiella pneumoniae* and other species of *Salmonella* to strongly induce chemokine expression [40—43]. Although induced to lower levels, *L. lactis* was able to stimulate all chemokines, except CCL5/RANTES, to significant levels ($p < 0.05$ to nil).

Of the chemokines induced by *L. lactis*, both CCL3/MIP-1α and CCL4/MIP-1β are ligands of the CCR5 chemokine receptor, which is preferentially expressed on Th1 cells [16]. A similar Gram-positive probiotic bacterium, *Lactobacillus rhamnosus*, was previously shown to induce mRNA expression of both these chemokines, amongst others, from primary macrophages derived from human blood [44]. That study also showed that culture supernatant of Lactobacilli-stimulated macrophages could enhance migration of Th1 cells. In our case, *L. lactis* was also found to induce expres-

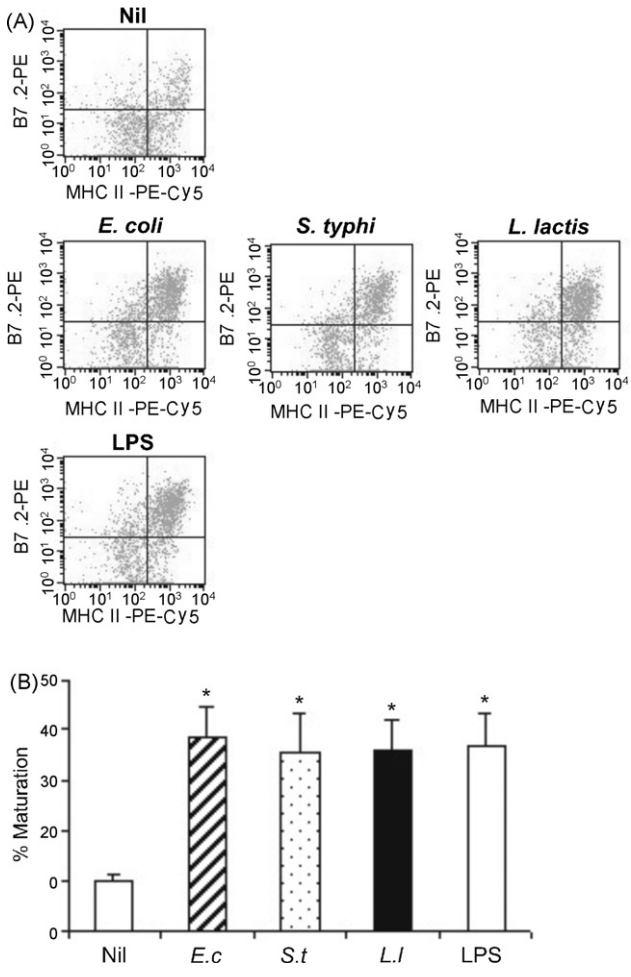

Figure 5 Induction of DC maturation by *E. coli*, *S. typhi* and *L. lactis in vitro*. Murine bone marrow derived DCs (10^6) were co-incubated overnight with 10^7 CFU of *L. lactis*, *E. coli* or *S. typhi*, left unstimulated (nil, negative control) or were stimulated with 1 μg/ml LPS (positive control). (A) Representative flow cytometry analyses of CD11c gated events, a DC marker are shown. Cells were stained for MHC-II and CD86 (B7.2) costimulatory molecule. (B) Percentage of mature DCs, double positive for MHC-II and CD86 surface markers, are shown. Data presented are the means and standard errors of three individual experiments; $^{*}p < 0.05$ to nil.

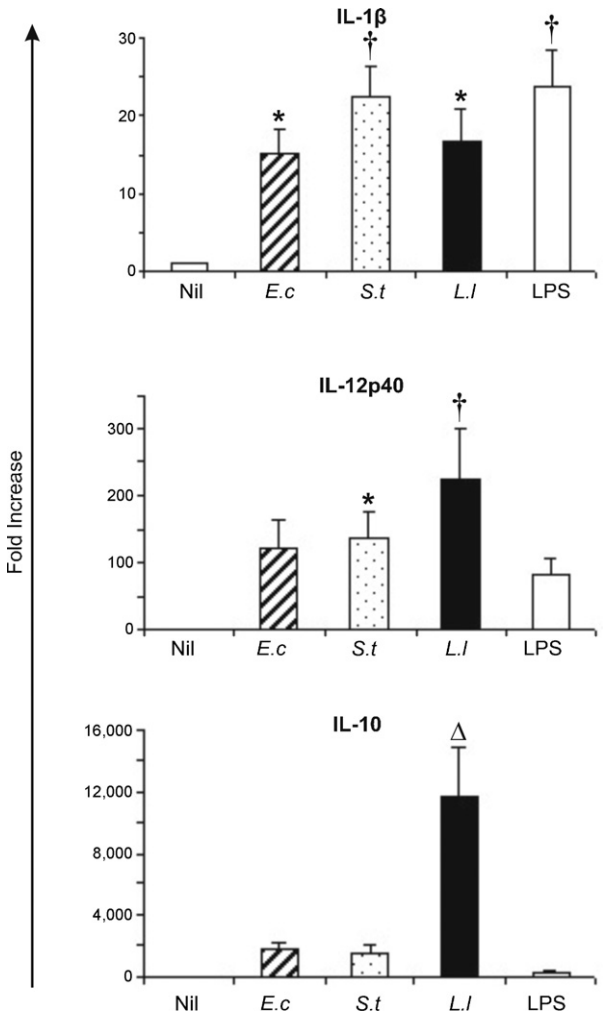

Figure 6 Induction of cytokine mRNA expression in DCs by *L. lactis*, *E. coli* and *S. typhi in vitro*. Murine bone marrow derived DCs (10^6) were co-incubated overnight with 10^7 CFU of *L. lactis*, *E. coli* or *S. typhi*, left unstimulated (nil, negative control) or were stimulated with 1 μg/ml LPS (positive control). CD11c+ events were sorted by FACS and cytokine mRNA was quantified by reverse-transcriptase qPCR. Levels of IL-1β, IL-12p40 and IL-10 mRNA were normalized to levels of GAPDH mRNA and presented as the fold increase from unstimulated (nil) negative control. Data presented are the means and standard errors of three individual experiments. $^{*}p < 0.05$ to nil; $^{\dagger}p < 0.01$ to nil; $^{\Delta}p < 0.0001$ to nil.

sion of a Th2 stimulating chemokine, CCL2/MCP-1. This suggests that *L. lactis* may induce a balanced Th1 and Th2 immune response.

In addition, we showed that *L. lactis*, *E. coli* and *S. typhi* are capable of recruiting immune cells into murine air-pouches *in vivo*. All three bacteria induced comparable levels of leukocyte recruitment, which were mainly neutrophils. These results are in line with a previous study that numerated leukocyte recruitment by *Salmonella enteritidis* using an air-pouch model [40]. Although *L. lactis* is a food grade bacterium and is non-pathogenic, we showed that *L. lactis* stimulates total leukocyte recruitment as efficiently as the other bacteria. This suggests that the capacity of *L. lactis* to recruit appropriate cells for an adaptive immune response is equivalent to *E. coli* and *S. typhi*.

We investigated the chemokine mRNA expression of the recruited leukocytes to evaluate the general activation of inflammatory responses. Chemokines expressed in recruited leukocytes exhibited a different profile than that observed with macrophages co-incubated with bacteria *in vitro*. In the context of the air-pouch assay, both resident phagocytes and epithelial cells lining the inside of the air-pouch are stimulated to produce chemokines, which will induce recruitment of additional leukocytes. We harvested the recruited leukocytes and examined their chemokine expression levels; therefore, we did not directly stimulate these cells, as we did with a pure culture of macrophages *in vitro*. We primarily recruited neutrophils in addition to eosinophils, monocytes

and lymphocytes; this difference in cell type accounts for the disparity in chemokine expression profiles observed.

Leukocytes recruited by all three bacteria expressed high levels of CCL3/MIP-1α, CCL4/MIP-1β and CXCL2/MIP-2 mRNA. Results with the CCL3/MIP-1α chemokine are consistent with a previous study that showed other Gram-negative (*Salmonella typhimurium* and *Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus aureus*) bacteria were capable of strongly stimulating the expression of this chemokine from human blood derived neutrophils [41]. Moreover, we observed the induction of exclusive chemokine expression profiles in recruited leukocytes for each bacterial stimulus. This indicates a difference in the activation status of recruited cells, and suggests that as live vaccines, *L. lactis*, *E. coli* and *S. typhi* may function differently.

The induction of murine DC maturation by *L. lactis*, *E. coli* and *S. typhi* was also shown in this study. All three bacterial stimuli significantly increased surface expression of MHC-II and CD86, which specify activated or mature DCs. These results are consistent with previous studies that demonstrated the induction of DC maturation by *L. lactis* [45,29,46], which together suggests that *L. lactis* is able to induce an adaptive immune response. This gives an additional indication of the adjuvanticity of *L. lactis*, which is beneficial if utilized as a live vaccine.

We showed that co-incubation of DCs with *L. lactis* induced mRNA expression of proinflammatory cytokines IL-1β and IL-12. These results are comparable to previous studies involving similar Gram-positive LAB [47—49]. IL-1β was previously demonstrated to be highly induced in DCs stimulated with *Lb. reuteri* [48] and in monocytes stimulated by various Gram-positive and Gram-negative bacteria [47]. *Lactobacillus* spp. were shown to induce IL-12 cytokine expression from DCs [48] and in particular, *Lb. acidophilus* and *Lb. paracasei* induced higher levels of IL-12 than several strains of *E. coli* [49]. However, it is well known that the effect of probiotic bacteria is strain dependent, and in contrast to our results, another study failed to show that a Gram-positive LAB, *Lb. casei*, and *E. coli* to be capable of inducing IL-12 from DCs [50]. Since the cytokines IL-1β and IL-12 are known to function as effective adjuvants [35,36], the induction of these cytokines by *L. lactis* suggests that this bacterium may exhibit adjuvant properties.

We also demonstrated that *L. lactis* can induce higher mRNA levels of IL-10 in DCs. This cytokine exhibits both anti-inflammatory and immunostimulatory activities [38]. Other Gram-positive bacteria including *Lactobacillus* and *Bifidobacterium* spp. were previously found to induce IL-10 cytokine from DCs, although in general, Gram-negative bacteria were found to induce expression of IL-10 to much higher levels [49]. Moreover, Mohamadzadeh et al. failed to show that any of the *Lactobacillus* spp. studied were able to induce expression of IL-10 from DCs [48]. Although similar characteristics are often attributed to all Gram-positive LAB, our results illustrate a unique cytokine induction profile from DCs stimulated with *L. lactis*. The induction of IL-10 mRNA suggests that *L. lactis* may play a homeostatic role by dampening inflammatory immune responses. Conversely, the induction of IL-10 by *L. lactis* suggests that this bacterium may function as an adjuvant by inducing humoral immune responses in addition to cellular responses resulting from the induction of IL-12.

In summary, we demonstrated that *L. lactis* triggers innate inflammatory responses: it induces chemokine mRNA expression *in vitro* and *in vivo*, stimulates DC maturation and cytokine mRNA expression. These results illustrate a capacity for adjuvanticity by *L. lactis*, and strengthen previous claims that *L. lactis* exhibits adjuvant effects. This study provides a basis for subsequent investigations to further elucidate the molecular mechanisms of *L. lactis* activation in the immune system. Moreover, we are currently investigating the adaptive immune responses of *L. lactis* live vaccines.

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