

Distinct gut-derived lactic acid bacteria elicit divergent dendritic cell-mediated NK cell responses

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Abstract

Lactic acid bacteria (LAB) are abundant in the gastrointestinal tract where they continuously regulate the immune system. NK cells are potently activated by dendritic cells (DCs) matured by inflammatory stimuli, and NK cells are present in the gut epithelium and in mesenteric lymph nodes, but it is not known how NK–DC interactions are affected by the predominantly non-pathogenic LAB. We demonstrate that human DCs exposed to different strains of gut-derived LAB consistently induce proliferation, cytotoxicity and activation markers in autologous NK cells. On the contrary, strains of LAB differ greatly in their ability to induce DC-dependent IFN- γ production by NK cells. This suggests that DCs stimulated by gut LAB may expand the pool of NK cells and increase their cytotoxic potential. Specific LAB, inducing high levels of IL-12 in DCs, may promote amplification of a type-1 response via potent stimulation of IFN- γ production in NK cells. Combining IFN- γ -inducing and non-inducing LAB completely abrogates DC-mediated IFN- γ production by NK cells, and therefore LAB modulating IFN- γ production in NK cells may be important regulators of the immune response.

Introduction

Mechanisms linking innate immune function with adaptive immunity are gaining interest, and much attention has been given to the interactions between myeloid dendritic cells (DCs) and NK cells. NK cells are capable of initiating cytokine production in the early phase of an immune response and kill cells expressing an altered MHC-repertoire and specific stress-induced molecules (1). The bidirectional crosstalk between NK cells and DCs includes induction of IFN- γ production (2, 3), activation markers (4), increased proliferation (5, 6) and cytotoxic activity (3, 7) in NK cells by mature DCs (mDCs), as well as maturation of immature DCs (iDCs) by NK cells (2, 4, 5). It has also been proposed that iDCs that are not successfully matured upon contact to NK cells are killed by activated NK cells (8), possibly permitting survival only of DCs that have encountered relevant antigen. Maturation of DCs by inflammatory mediators such as tumour necrosis factor (TNF)- α (3, 6) or toll-like receptor (TLR) ligands (2, 7) has been widely used in the study of DC–NK interactions. In addition, maturation by intact pathogens confers NK cell-stimulatory

activity to DCs, as has been shown for *Escherichia coli*, *Mycobacterium bovis* BCG (9) and *Helicobacter pylori* (10).

Interactions between NK cells and DCs are likely to occur in the gut-associated lymphoid tissue, where NK cells reside among intra-epithelial lymphocytes (11), or in the mesenteric lymph nodes through which gut DCs continuously circulate (12). The interaction between antigen-loaded mDCs and NK cells in lymph nodes has been described to result in prompt IFN- γ production, and to be necessary for T_H1 polarization of subsequent adaptive responses (13, 14), and this may also apply to the gut-associated immune system. DCs are important gatekeepers in the intestine. They reside in Peyer's patches, capturing antigen shuttled through M-cells (15) and in the lamina propria where they sample antigen that cross the epithelial barrier or send dendrites through tight junctions of the epithelium to sense antigen in the intestinal lumen (16). Therefore, these cells are considered important in the diverse responses to pathogens versus harmless food antigens and commensal gut microorganisms elicited in the

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healthy intestine. Accumulating evidence shows that commensal bacteria play a role in educating immune cells of the gut, including DCs, to induce appropriate responses to intestinal antigens (15).

Lactic acid bacteria (LAB) comprise several genera of which lactobacilli, bifidobacteria and enterococci are abundant in the intestinal tract; especially in the lower small intestine and colon (17). LAB are widely used in fermented foods, wherein they are termed 'probiotic' when considered beneficial to health (18). One potential health-promoting property is modulation of the immune system. It has been previously demonstrated that different LAB strains induce variable levels of co-stimulatory molecules and cytokine release in murine and human DCs (19, 20). Certain strains of *Lactobacillus* species added during DC maturation have been shown to lead to DCs that induce Tr, whereas other strains do not (21). DCs matured by three specific *Lactobacillus* strains induce IFN- γ production in T cells (22), while DCs matured by a *Lactobacillus plantarum* strain were able to skew T cells from allergic patients away from a T_H2 cytokine response *ex vivo* (23). Thus, distinct LAB possess the ability to polarize DCs towards T_H1- or Tr-inducing phenotypes, and this may be the reason for beneficial effects observed following LAB administration in patients suffering from infectious diarrhoea and atopic diseases (24). Nevertheless, mechanistic evidence for these effects is lacking.

An enhancement of NK cell activity after LAB consumption has been observed in a number of human intervention studies in healthy adults (25), elderly (26–29) and smokers (30). When mice are fed LAB concomitantly with mutagen injections, increased NK cytolytic activity correlates with a decrease in tumour incidence, and the positive effect of probiotic supplementation is absent in NK cell-deficient beige mice (31). A plausible explanation for the observed increase in NK cytolytic activity after intake of certain probiotic formulations is that NK cells interact directly with the non-pathogenic LAB or with DCs stimulated by LAB. The aim of this study was to characterize NK–DC interactions in response to gut-derived LAB having differential effects on DCs. We report that DCs matured by LAB consistently induce activation and promote proliferation and cytotoxicity in NK cells and that strains of different LAB species differ importantly in their capacity to induce IFN- γ production in NK cells via DCs.

Methods

Cell isolation and culture

PBMCs were obtained from buffy coats (Copenhagen University Hospital, Denmark, and G. Gaslini Institute, Genoa, Italy) by centrifugation on Ficoll–Paque separation medium. Cells were cultured in RPMI 1640 containing 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin (all from Cambrex Bio Science, East Rutherford, NJ, USA) referred to as complete medium. CD14⁺ cells were isolated from PBMC using anti-CD14 microbeads (Miltenyi, Bergisch Gladbach, Germany) and differentiated into iDCs during 6 days of culture in a humidified 5% CO₂ atmosphere in complete medium

supplemented with 20 ng ml⁻¹ granulocyte macrophage colony-stimulating factor (Biosource, Camarillo, CA, USA) and 100 IU ml⁻¹ IL-4 (Euroclone, Milan, Italy). Fresh medium containing cytokines was supplied on day 3 of culture. DCs were incubated with UV-killed LAB (25 μ g per 10⁶ DCs ml⁻¹) in 24-well plates for 6 or 20 h to yield mDCs. NK cells were negatively isolated from PBMC using the NK Cell Isolation Kit II (Miltenyi). Isolated NK cells were consistently >95% CD3⁻CD56⁺ cells and devoid of monocytes. NK cells (10⁵ cells per well) were cultured with autologous DCs (2 \times 10⁴ cells per well) with or without LAB (0.5 μ g per 2 \times 10⁴ DCs 0.2 ml⁻¹) in round-bottom 96-well plates. IL-12 neutralization antibody (2 μ g ml⁻¹, R&D Systems, Minneapolis, MN, USA) or IL-10 neutralization antibody (2.5 μ g ml⁻¹, BD Biosciences, Franklin Lakes, NJ, USA) were added to cultures where indicated.

Bacterial strains

The strains of LAB used were *Lactobacillus reuteri* DSM12246, originally isolated from pig faeces (32), and *Lactobacillus acidophilus* X37 and *Bifidobacterium bifidum* S13.1, isolated from a human intestinal biopsy and from a child's faeces, respectively. The latter two strains were isolated and typed at the Department of Food Science, University of Copenhagen, Denmark. LAB were grown in de Man, Rogosa and Sharpe medium (Merck, Darmstadt, Germany) until stationary growth phase, harvested by centrifugation at 2000 \times g for 15 min, washed twice with sterile PBS, re-suspended in PBS and UV irradiated for 15 min in a thin film of liquid. Dry matter determinations were made in quadruplicates. LPS contamination of LAB suspensions was tested with the Pyrochrome Kit (Association of Cape Cod, East Falmouth, MA, USA). Endotoxin content was <0.1 EU ml⁻¹ for all LAB suspensions at the concentrations used in cell culture experiments. UV-killed LAB were stored at -80°C. To examine compounds released during bacterial growth, LAB were grown in antibiotics-free complete medium until stationary growth phase, centrifuged at 2000 \times g and the supernatant sterile filtered (0.2 μ m). Filtered supernatants were added to DCs at 10% of the culture volume.

Cytokine quantification

Cytokine concentrations in culture supernatants were determined using ELISA kits for IL-12p70 (R&D Systems) and IFN- γ (Biosource) according to the manufacturers' instructions. IL-10 was quantified by ELISA using a mAB pair from BD Biosciences (clones: JES319F1 and biotinylated JES3-12G8).

Cell surface marker analysis

Cells were incubated with human γ -Ig (human therapy grade) for 10 min at room temperature to prevent binding of staining antibodies to Fc receptors. Subsequently, cells were incubated for 30 min at 4°C with unconjugated mABs against HLA-I (clone W6/32, kindly provided by M.C. Mingari, University of Genoa, Italy), HLA-DR (clone D12.1, kindly provided by G. Frumento, IST, Genoa, Italy) or CCR7 (BD Biosciences) followed by staining with appropriate FITC-labelled secondary antibodies, with FITC-labelled mABs against CD3 (BD Biosciences), CD80 and CD83 (both Beckman

Coulter, Fullerton, CA, USA), PE-labelled mABs against CD25, HLA-DR (both BD Biosciences), CD69 (Ancell, Bayport, MN, USA) and NKp44 (Beckman Coulter) and/or with a PE-Cy5-labelled mAB against CD56 (Beckman Coulter). Propidium iodide (Sigma, St Louis, MO, USA) was used to assess cell viability. Flow cytometric analysis of surface marker expression was performed on a FACSScan flow cytometer (BD Biosciences).

Proliferation assay

Cell proliferation during 4 days of co-culture of DCs and NK cells was assessed by adding tritiated thymidine (Amersham Biosciences) to the cultures 20 h prior to harvesting them onto glass filter mats using a cell harvester (AQS Manufacturing, Horsham, UK). Incorporation of tritiated thymidine was quantified in a scintillation counter (Packard, Meriden, CT, USA). Results are given as counts per minute (c.p.m.).

Cytotoxicity assay

NK cell cytotoxicity against the target cell line K562 was assessed in a 4-h Cr^{51} release assay. NK cells and Cr^{51} -labelled target cells were co-cultured in V-bottom 96-well plates at the indicated ratios for 4 h at 37°C. Aliquots of labelled target cells were incubated without NK cells to quantify spontaneous Cr^{51} release or lysed with 2 N HCl to assess total Cr^{51} release. Cr^{51} released into the supernatant was quantified in a gamma counter (Beckman Coulter). The percentage of specifically lysed target cells was calculated as $100 \times (\text{sample release} - \text{spontaneous release}) / (\text{total release} - \text{spontaneous release})$.

Statistical analysis

Statistical analysis (one-way or two-way analysis of variance with Bonferroni *post hoc* test) was performed using the GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA, USA). Differences were considered significant if $P < 0.05$.

Results

Gut-derived LAB induce species-dependent cytokine patterns in DCs and DC-dependent IFN- γ release by NK cells

We have previously observed great differences in the amount of IL-12 produced by DCs in response to different single and combined strains of LAB (19, 20). Prior to investigating the effect of LAB-stimulated DCs on NK cells, we assessed the cytokine pattern of DCs cultured in the presence of single and combinations of three representative strains of gut-derived LAB. In accordance with the earlier results, monocyte-derived iDCs cultured with UV-killed LAB for 20 h produced different amounts of IL-12 and IL-10 (Fig. 1A). *Lactobacillus acidophilus* induced the highest amount of IL-12 and slightly more IL-10 than the other bacteria. Adding a combination of *L. acidophilus* and *B. bifidum* or *L. reuteri* to iDCs abrogated IL-12 production, whereas IL-10 production was additively increased.

The high ratio of IL-10 to IL-12 induced by *B. bifidum* and *L. reuteri* suggested that IL-10 is responsible for the inhibition of *L. acidophilus*-induced IL-12 production. When

L. acidophilus and an IL-12-inhibitory LAB strain were added together with an anti-IL-10 antibody, IL-12 production was indeed markedly increased (Fig. 1B, left panel), but neutralizing IL-10 also substantially increased IL-12 production induced by *L. acidophilus*, *B. bifidum* or *L. reuteri* added separately. The inhibitory effect of *B. bifidum* and *L. reuteri* on *L. acidophilus*-induced IL-12 production was maintained with increasing concentrations of anti-IL-10 antibody (Fig. 1B, right panel).

When iDCs and individual or combinations of LAB strains were added simultaneously to NK cells, the IFN- γ production by NK cells (Fig. 1C, left panel) correlated with the amount of IL-12 induced in DCs. As expected, *L. acidophilus*-induced IFN- γ production was significantly reduced when IL-12 was neutralized by an anti-IL-12 antibody, whereas addition of anti-IL-10 antibody slightly increased IFN- γ production. The IFN- γ production upon IL-10 neutralization was, however, not increased to a degree matching the increase in DC IL-12 production. NK cells stimulated with different strains of LAB in the absence of DCs did not produce IFN- γ . Large amounts of TNF- α were produced in the NK-DC co-cultures stimulated with LAB, especially with *L. acidophilus*, but TNF- α was mainly produced by DCs and NK cells did not contribute significantly (not shown).

To examine the nature of the bacterial stimuli evoking the differential cytokine production in DCs and NK cells, we cultured LAB in cell culture medium until stationary growth phase and sterile filtered the supernatant. Adding 10% supernatant of *L. acidophilus*, *B. bifidum* or *L. reuteri* to iDCs during 20 h of culture did not induce cytokine production, indicating that the presence of intact bacteria is required for induction of IL-12 and IL-10 (Fig. 2). Conversely, combining UV-killed *L. acidophilus* bacteria with sterile-filtered supernatant of *B. bifidum* or *L. reuteri* diminished IL-12 production by DCs markedly compared with the addition of *L. acidophilus* alone, suggesting that inhibitory components of these bacteria are released into the growth medium.

LAB with different cytokine-inducing properties deliver maturation stimuli to DCs, and DCs matured by LAB induce expression of activation markers on NK cells

Kinetics studies showed that peak *L. acidophilus*-induced IL-12 production occurred after only 6 h of stimulation of DCs with LAB (data not shown). During this incubation period, all three strains of LAB increased expression of the maturation markers CD80, CD83, CD86 and HLA-DR on DCs, *L. acidophilus* and *B. bifidum* more than *L. reuteri* (Fig. 3). This indicates that low or absence of IL-12 production by DCs is not paralleled by a complete lack of maturation. Expression of HLA class I was slightly increased in DCs after 6 h of culture with LAB, and the expression increased further during prolonged incubation (Fig. 3). The chemokine receptor necessary for migration to lymph nodes, CCR7, was absent on DCs after 6 h of culture with LAB but induced to a variable extent by the different LAB strains after 20 h of culture (Fig. 3).

Since extensive up-regulation of maturation markers occurred after only 6 h of incubation with LAB, DCs were matured for 6 h (followed by washing to remove non-phagocytosed

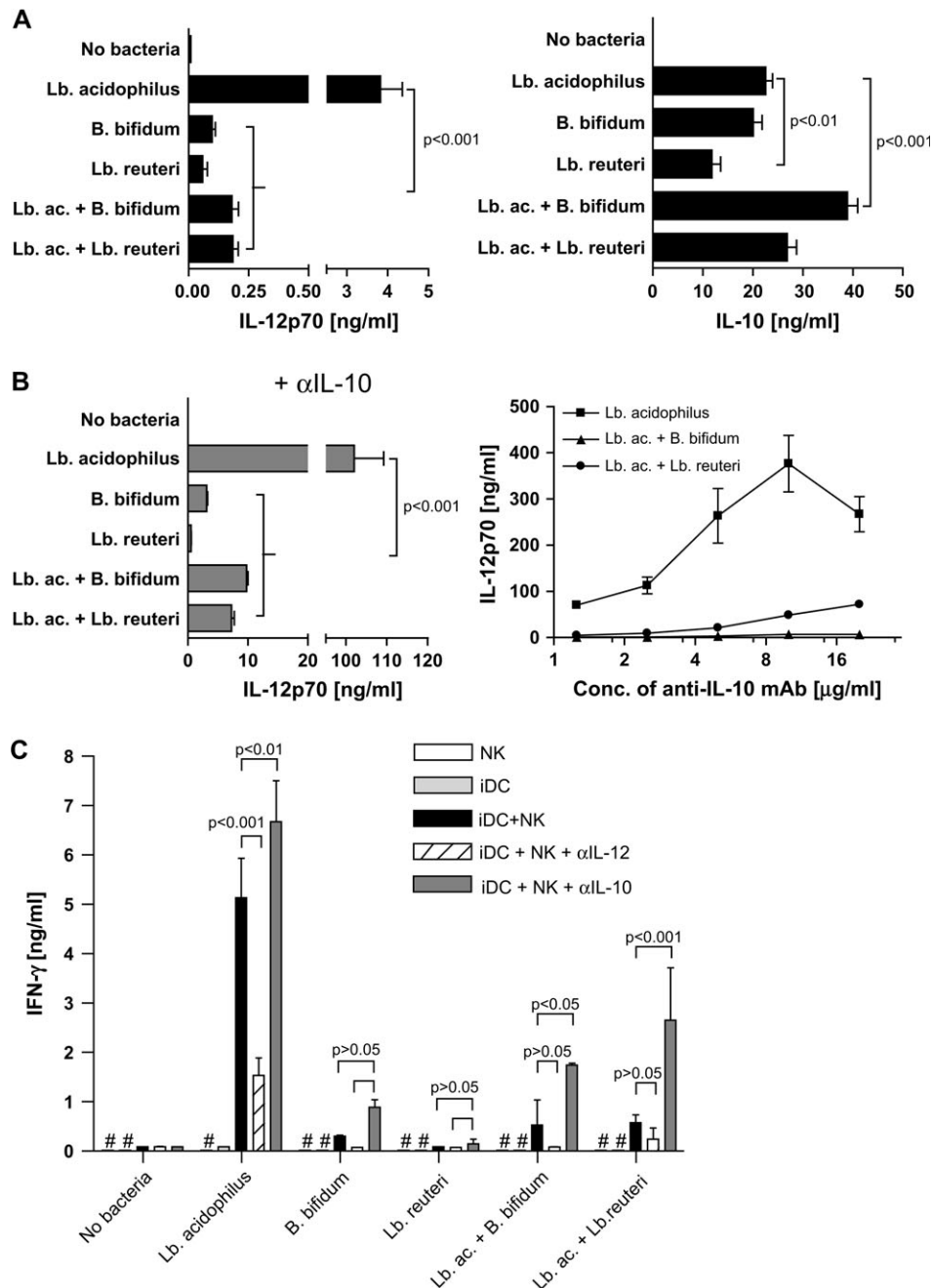


Fig. 1. Distinct LAB induce different amounts of cytokines in DCs and NK cells. (A) UV-killed LAB (*Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Lactobacillus reuteri* or *L. acidophilus* combined with one of the other strains; $25 \mu\text{g}$ per 10^6 DCs ml^{-1} , 20 h) induced production of variable amounts of IL-12 and IL-10 in iDCs. (B) IL-12 production by iDCs cultured with LAB increased for all stimuli upon addition of IL-10 neutralization antibody, but the ability of *B. bifidum* and *L. reuteri* to reduce *L. acidophilus*-induced IL-12 production was maintained with increased concentrations of anti-IL-10 antibody. (C) UV-killed LAB (*L. acidophilus*, *B. bifidum*, *L. reuteri* or *L. acidophilus* combined with one of the other strains; $0.5 \mu\text{g}$ per 2×10^4 DCs per 0.2 ml^{-1} , 48 h) induced IFN- γ production in NK cells (10^5 per well) cultured with autologous iDCs. IFN- γ production was reduced upon addition of anti-IL-12 antibody, but slightly increased when anti-IL-10 antibody was added. Cytokines in culture supernatants were quantified by ELISA. Data are means and SDs of duplicate cultures. 'Hash' indicates not detected. Data are representative of four experiments with cells from different donors.

bacteria and secreted cytokines) for further studies of NK cell activation. DCs matured by all three strains of LAB and combinations thereof induced expression of several surface markers indicative of NK cell activation (Fig. 4A). Expression of CD25, the IL-2 receptor α -chain, indicates high responsiveness to

IL-2. DCs matured by all LAB caused up-regulation of CD25 on NK cells, with *L. acidophilus*-matured DCs being the most potent. CD56 is present on all resting NK cells, but to a lesser extent on CD16⁺ NK cells, which is the more cytolytic subset of NK cells (33). Upon activation of these CD56^{dim} cells,

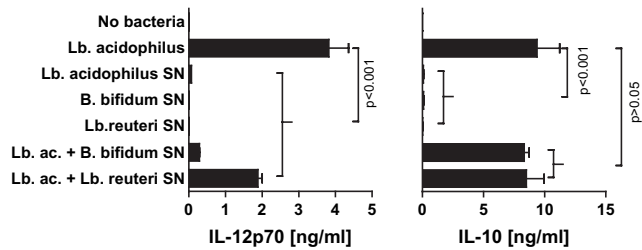


Fig. 2. Induction of IL-12 production requires presence of bacteria, whereas inhibition of IL-12 production is induced by bacteria-derived soluble or shed factors. Sterile-filtered supernatant (SN) from LAB grown in cell culture medium (*Lactobacillus acidophilus*, *Bifidobacterium bifidum* or *Lactobacillus reuteri*, 10% v/v) did not induce IL-12 or IL-10 production in iDCs during 20 h of culture, but supernatant of *B. bifidum* and *L. reuteri* suppressed IL-12 production induced by UV-killed *L. acidophilus* (25 μg per 10^6 DCs ml^{-1}) without affecting IL-10 production. Data are means and SDs of duplicate cultures. Data are representative of three experiments with cells from different donors.

CD56 is up-regulated to the level present on resting CD16⁻CD56^{bright} NK cells. DCs matured by all LAB efficiently up-regulated CD56 on CD56^{dimm} NK cells. CD69 is a receptor for unknown ligands known as ‘early activation marker’ on NK cells and T cells (34). CD69 was up-regulated on NK cells cultured with all LAB-matured DCs, but to a lesser extent by DCs matured by *B. bifidum* or *L. reuteri*. DCs matured by *B. bifidum* or *L. reuteri* combined with *L. acidophilus* also induced a slightly lower expression of CD69 in NK cells than DCs matured by *L. acidophilus* alone. HLA-DR is expressed on NK cells upon activation and is possibly functional in antigen presentation (35). HLA-DR was up-regulated on NK cells by LAB-matured DCs, especially by DCs matured by *B. bifidum* or *L. reuteri* both with and without the simultaneous presence of *L. acidophilus*. Finally, NKp44 is one of the three identified orphan natural cytotoxicity receptors (NCRs) correlated with NK cell cytotoxicity and the only NCR not constitutively expressed but induced upon activation of NK cells (36). All LAB-matured DCs induced expression of NKp44 on a subset of NK cells.

We next analysed whether 6 h of contact with LAB allowed DCs to modulate NK cell IFN- γ production. As expected, IFN- γ production was mainly induced in NK cells co-cultured with DCs previously matured by *L. acidophilus* (Fig. 4B), and neutralizing IL-12 reduced IFN- γ production, indicating that IL-12 produced by mDCs was largely responsible for induction of IFN- γ production in NK cells. Similar to the simultaneous addition of iDCs and LAB to NK cells (Fig. 1B), addition of DCs matured for 6 h with *B. bifidum*, *L. reuteri* or one of these combined with *L. acidophilus* induced less IFN- γ in NK cells than *L. acidophilus*-matured DCs (Fig. 4B).

DCs matured by all LAB expand NK cells and increase their cytolytic capacity

NK cell proliferation during 4 days of culture was increased by DCs matured for 6 h by the different strains of LAB and combinations thereof (Fig. 5A). As previously described (9), mDCs augmented proliferation significantly more than iDCs. mDCs alone or NK cells cultured separately with UV-killed bacteria did not proliferate (<100 c.p.m., not shown). DCs in

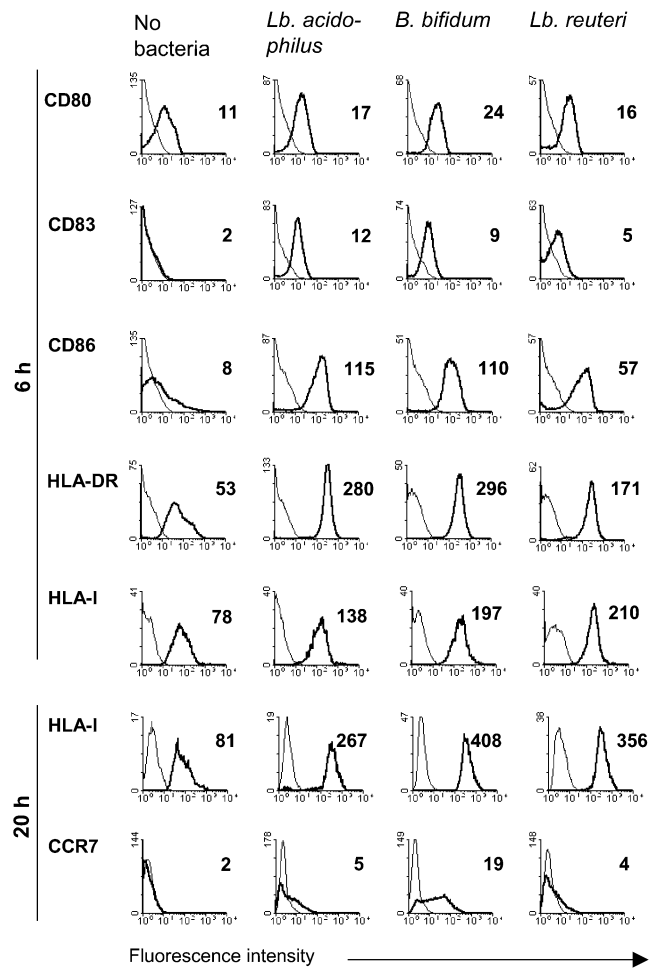


Fig. 3. Short-term culture with LAB induces maturation of DCs. Expression of surface maturation markers was measured after culture of iDCs with no bacteria or with UV-killed LAB (*Lactobacillus acidophilus*, *Bifidobacterium bifidum* or *Lactobacillus reuteri*, 25 μg per 10^6 DCs ml^{-1}) for the indicated number of hours. Thin lines depict cells stained with an isotype control antibody; bold lines depict cells stained with an antibody specific for the surface marker indicated. Numbers in bold indicate the geometrical mean fluorescence intensity of cells stained with the specific antibody. Data are representative of four experiments with cells from different donors.

general were seen to promote survival of NK cells as the NK cell viability percentage was increased almost 2-fold in the presence of DCs stimulated or not by bacteria (data not shown). The proliferation and viability measures suggest that bacterially matured DCs increased the amount of viable NK cells in the cultures. In addition, DCs matured by the different LAB increased NK cell cytotoxicity against the target cell line K562 after a 48-h co-culture of DCs and NK cells, beyond the increase seen with iDCs (Fig. 5B).

Discussion

LAB are known to be involved in the maintenance of gut immune homeostasis, and now also emerge as potential NK cell modulators. All LAB tested in the present study mediated, via DC maturation, expansion of NK cells and increased their cytotoxic efficacy as well as the level of

cytotoxicity-related activation markers. This indicates that LAB, similar to pathogenic microorganisms and inflammatory stimuli, license DCs to signal to NK cells. Our data suggest that stimulation of NK cell proliferation and cytotoxicity is a general ability of LAB. In support of this finding, the increased NK cell activity shown in mice and humans upon consumption of lactobacilli and bifidobacteria was obtained using a number of different species and strains (27–31). An enlarged and more cytolytic pool of NK cells would be

beneficial prophylactically in healthy individuals but also therapeutically in most pathologies. Moreover, as NK cell cytotoxicity is tightly controlled by the presence of NK cell receptor ligands on target cells (1), an expansion of NK cells caused by gut LAB is not likely to cause excess tissue damage.

In the present study, IL-12 production in DCs was strongly induced by *L. acidophilus*, whereas all LAB tested induced substantial amounts of IL-10. Other LAB strains have been found to elicit highly variable levels of these cytokines (22, 23). Only DCs matured by *L. acidophilus* induced high amounts of IFN- γ in NK cells, suggesting that not all LAB have this capability. It is generally accepted that IL-12 induces IFN- γ production in human NK cells (13), as it is also the case in our study. Recent findings indicate that IFN- γ production by NK cells is required to induce T_H1 responses in lymph nodes (14), emphasizing the importance of bacterial regulation of IL-12 production in DCs. It is not known how *L. acidophilus* induces IL-12 production in DCs. However, as *L. acidophilus* supernatant was unable to induce IL-12 production, this stimulation is hypothesized to involve a cell wall component, possibly acting via TLRs on DCs. This would be in accordance with Michelsen *et al.* (37), showing that maturation and IL-12 production can be induced in murine DCs via TLR2 recognizing peptidoglycan and lipoteichoic acid. Amounts of TLR2 ligands expressed by the different LAB may be the reason for the observed differences in induction of maturation surface markers in monocyte-derived DCs cultured with LAB for 6 h. In agreement with our previous results (19, 20), *B. bifidum* and *L. reuteri* were found to inhibit *L. acidophilus*-induced IL-12 production in DCs, and accordingly abrogated IFN- γ production by NK cells. A similar mechanism may cause a probiotic mixture of eight strains of LAB, VSL#3, to induce only IL-10 production in murine DCs (38). This dominant IL-12-inhibitory property of a mixture of LAB may be of importance in the intestine where thousands of strains co-exist. The inhibitory components of these bacteria are seemingly secreted or 'shed' into the medium during growth, perhaps indicating that such compounds reach gut DC compartments that intact bacteria do not normally access. In our study, we found that IL-10 played a role in limiting the amount of IL-12 produced in response to LAB, as neutralizing IL-10 increased the IL-12

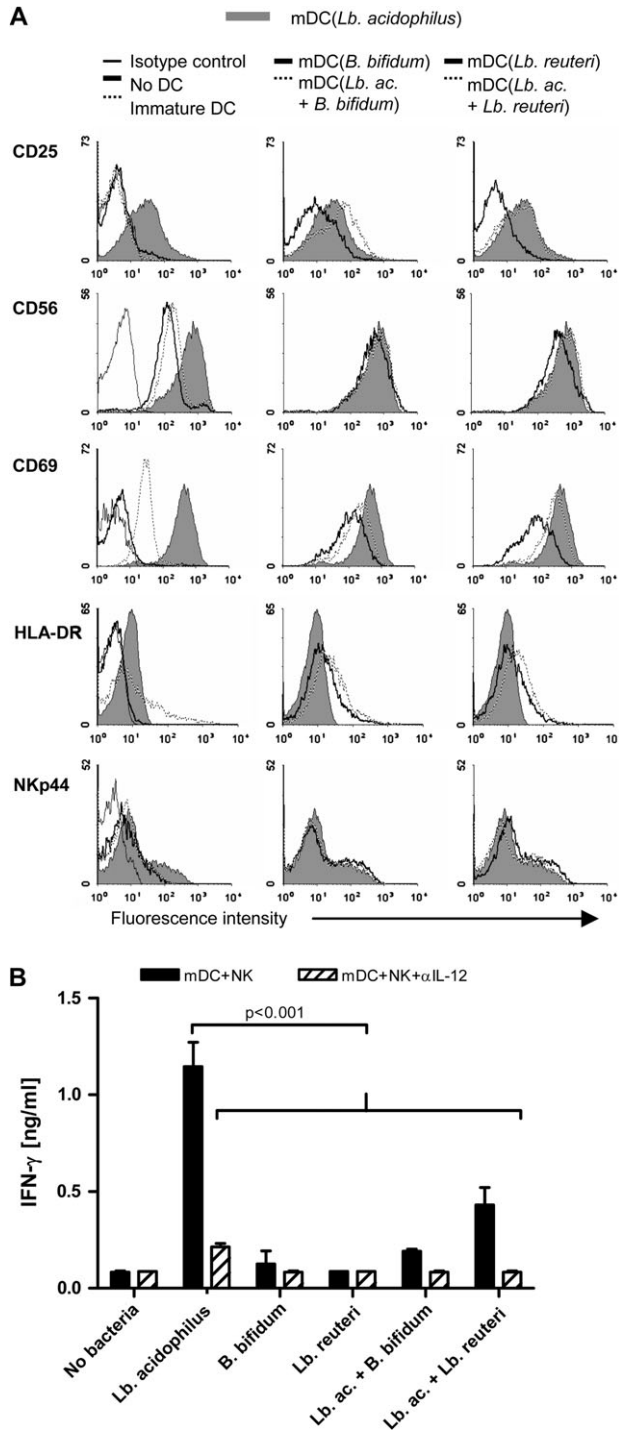


Fig. 4. LAB-matured DCs induce species-dependent activation and IFN- γ production in NK cells. (A) Expression of activation markers on NK cells was increased after culture with autologous DCs matured for 6 h with UV-killed LAB. In all panels, filled histograms depict NK cells cultured for 48 h with DCs matured by *Lactobacillus acidophilus*. Thin lines depict cells stained with an isotype-matched control antibody, and bold or dotted lines depict NK cells cultured with or without different DCs (mDC, DC matured by the bacteria indicated) and stained with an antibody specific for the surface molecule indicated. Data are representative of two independent experiments. (B) NK cells (10^5 per well) produced IFN- γ when incubated for 48 h with autologous DCs (mDCs, 2×10^4 per well) matured for 6 h with UV-killed LAB (*L. acidophilus*, *Bifidobacterium bifidum*, *Lactobacillus reuteri* or *L. acidophilus* combined with one of the other strains; $25 \mu\text{g per } 10^6$ DCs ml^{-1}). IFN- γ production was reduced in the presence of an IL-12 neutralization antibody. IFN- γ in culture supernatants was quantified by ELISA. Data are means and SDs of duplicate cultures. Data are representative of four experiments with cells from different donors.

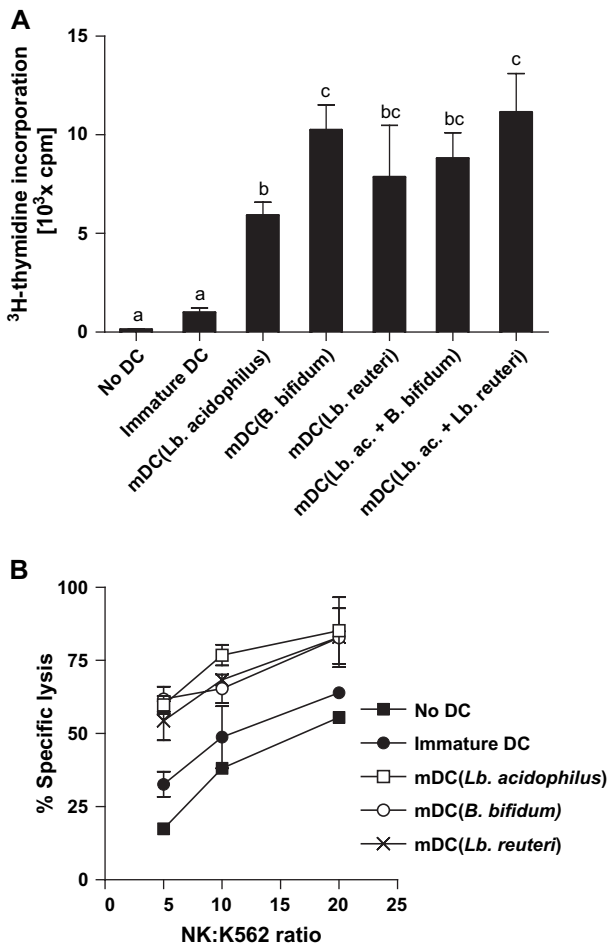


Fig. 5. LAB-matured DCs increase proliferation and cytotoxicity in NK cells. (A) NK cells (10^5 per well) proliferated less when incubated for 4 days with autologous iDCs (2×10^4 per well) than with mDCs, DC matured for 6 h with UV-killed LAB (*Lactobacillus acidophilus*, *Bifidobacterium bifidum*, *Lactobacillus reuteri* or *L. acidophilus* combined with one of the other strains; $25 \mu\text{g}$ per 10^6 DCs ml^{-1}), followed by washing to remove bacteria. Proliferation data are means and SDs of c.p.m. values of quadruplicate culture wells and are representative of independent experiments with cells from three donors. Bars labelled with different letters indicate significantly different values ($P < 0.01$). Bars labelled with the same letters indicate that values are not significantly different ($P > 0.05$). (B) NK cells became more cytolytic after culture with DCs. NK cells (10^5 per well) were cultured for 48 h in 48-well plates in the presence of autologous iDCs or mDCs (10^5 per well) prematured by the indicated LAB strains for 6 h ($25 \mu\text{g}$ per 10^6 DCs ml^{-1}). Subsequently, 4 h cytotoxicity against the NK cell target K562 cell line was assessed by Cr^{51} release. Data are means and SDs of triplicate measurements and are representative of three experiments with cells from different donors.

production induced by all single and combined LAB strains. It is known that IL-10 inhibits IL-12 production in DCs in an autocrine fashion by regulating IL-10 receptor expression and activating STAT-3 (39). Nevertheless, IL-10 was not responsible for the inhibition of *L. acidophilus*-induced IL-12 production by *B. bifidum* and *L. reuteri*, as this inhibition was also evident in the presence of IL-10-blocking antibody, and when *B. bifidum* and *L. reuteri* were replaced by their non-IL-10-inducing supernatant. Therefore, it is unlikely that the secreted components of *B. bifidum* and *L. reuteri*, exerting

the IL-12 inhibitory effect, are merely strong inducers of IL-10. Probably, they interact directly with inhibitory receptors on DCs, such as DC-specific intercellular adhesion molecule 3-grabbing non-integrin recognizing carbohydrate structures expressed, e.g. by *H. pylori* and mycobacteria, enabling their immune escape (40).

DCs matured by the different strains of LAB all induced activation markers in NK cells. CD56 and NKp44 were induced to a comparable extent by all strains, whereas CD25 was induced to a greater extent by *L. acidophilus* and HLA-DR by *B. bifidum* and *L. reuteri*. Similar to the IFN- γ production, the level of CD69 expression on NK cells seemed to be dominated by *B. bifidum* and *L. reuteri* when these bacteria were present together with *L. acidophilus* during maturation of DCs, as CD69 expression was reduced in NK cells stimulated with DCs matured by *L. acidophilus* together with *B. bifidum* or *L. reuteri*, compared with NK cells stimulated by DCs matured by *L. acidophilus* alone. Expression of CD69 has been shown to correlate with NK cell cytotoxicity (41), but its expression on NK and T cells has also been implicated in immune down-regulation (42), possibly underlining the regulatory properties of *B. bifidum* and *L. reuteri*. Regarding HLA-DR expression on NK cells, combining two strains of bacteria for DC maturation yielded a small additive effect, but as the role of NK cells as antigen-presenting cells remains ill-defined it is difficult to interpret the functional significance of such differences. Presumably, the cytokines and NK cell ligands expressed by LAB-matured DCs, which are responsible for induction of activation markers, increased cytotoxicity, and proliferation in NK cells do not differ importantly from NK-activating molecules secreted by DCs matured with LPS or pro-inflammatory cytokines. These include type I IFNs, IL-2, IL-15, IL-18, CD40 and CD80/CD86 [reviewed in ref. 43, 44].

In this study, we observed no direct stimulation of highly purified NK cells by any of the LAB analysed in the absence of DCs. NK cells have previously been shown to be the lymphocyte population most sensitive to activation by LAB, but only in the presence of monocytes (45, 46). These observations, however, do not rule out that NK cells in the gut may be able to directly detect LAB, possibly by interaction between bacterial CpG DNA and TLR9, which is present in NK cells (7, 47). We addressed this by testing the direct effect of LAB stimulation on both resting and polyclonally activated NK cells, but observed no IFN- γ production (not shown), so the LAB used in this study seemingly do not interact with TLR9 on NK cells, but rather with TLR2 on accessory cells as discussed above. TLR2 has been shown to be absent or expressed in low amounts in NK cells (48, 49), and its ligation only activates NK cells in the presence of exogenous IL-12 (49).

We chose the shortest time period of LAB exposure yielding mDCs, 6 h, for the co-culture experiments. However, CCR7 was not up-regulated until after 20 h of maturation by LAB. This may indicate that migration of DCs from the lamina propria or Peyer's patches to mesenteric lymph nodes does not occur before prolonged exposure to LAB or simultaneous exposure to other maturation stimuli have taken place. HLA-I molecules may protect against lysis by NK cells during migration, as HLA-I-deficient DCs are efficiently

killed by autologous NK cells, at least *in vitro* (8). Also in this study, we observed that more iDCs than LAB-matured DCs were lysed by activated NK cells (not shown). Lymph nodes have been identified as one of the sites where NK cells and DCs encounter (13, 14, 50), and it is likely that gut-derived DCs reach mesenteric lymph nodes upon LAB stimulation and acquisition of CCR7, and engage in crosstalk with resident NK cells in the paracortex, where concomitant CD4⁺ T cell activation takes place (13, 50). Therefore, NK cells interacting with migrating DCs may regulate co-localized T cell responses.

In conclusion, LAB potently initiate NK–DC interactions via DC maturation. NK cells expand and increase their cytolytic potential. The balance between NK cell responses and regulatory responses may prove delicately regulated by intestinal LAB, as NK cell effector functions are subjected to suppression mediated by Trs (51), and these Trs may be induced by LAB (21), which at the same time sustain NK cell cytolytic activity. However, in contrast to other DC maturation stimuli, different LAB have varying effects on NK cell IFN- γ production. Since T_H1-promoting LAB can easily be identified in this model system, these LAB may represent a useful tool for modulating the cytokine balance during autoimmune diseases driven by T_HIL-17 cells in the absence of IFN- γ (52), and to promote potent type-1 immune responses, desirable in infection and cancer. In addition, the presence of LAB early in life skewing the immune system towards a T_H1 response, possibly through the intermediate of NK cells, may aid in the prevention of T_H2-mediated allergy. Finally, the evidence that weak IFN- γ -inducing LAB strains are able to suppress the action of IFN- γ -inducing strains while preserving NK cell-stimulatory activity could represent a pivotal mechanism in maintaining immunological homeostasis in the intestine.

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Abbreviations

c.p.m.	counts per minute
DC	dendritic cell
iDC	immature DC
LAB	lactic acid bacteria
mDC	mature DC
NCR	natural cytotoxicity receptor
TLR	toll-like receptor
TNF	tumour necrosis factor

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