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ORIGINAL RESEARCH PAPER

Production of biologically active human lymphotactin (XCL1) by *Lactococcus lactis*

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Abstract Lymphotactin-XCL1 is a chemokine produced mainly by activated CD8+ T-cells and directs migration of CD4+ and CD8+ lymphocytes and natural killer (NK) cells. We expressed human lymphotactin (LTN) by the lactic-acid bacterium *Lactococcus lactis*. Biological activity of LTN was confirmed by chemo-attraction of human T-cells by chemotaxis demonstrating, for the first time, how this chemokine secreted by a food-grade prokaryote retains biological activity and chemoattracts T lymphocytes. This strain thus represents a feasible well-tolerated vector to deliver active LTN at a mucosal level.

Keywords Biological activity · *Lactococcus lactis* · Lymphotactin · Secretion

Laura M. Zavala-Flores and Julio Villatoro-Hernandez have contributed equally to this work.

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Introduction

Chemokines, the largest family of cytokines, cause leukocyte mobilization through chemotaxis at sites of antigenic challenge or lesions. Lymphotactin (LTN), the only member of the C subfamily of chemokines, is mainly secreted by activated T lymphocytes and it chemoattracts CD4+ and CD8+ lymphocytes as well as natural killer (NK) cells (Hedrick et al. 1997). This chemokine plays an important role in maintaining the integrity of the epithelium and in mucosal immune responses. Previous studies revealed that the intranasal administration of LTN resulted in the enhancement of the mucosal and systemic antibody response through help provided by Th1- and Th2-type cells (Lillard et al. 1999).

Moreover, this chemokine has some antitumor activity and, when expressed in engineered myeloma

cells, resulted in tumor regression in mice. This effect was assumed to be mediated by CD4+ and CD8+ cells and neutrophils expressing the XCR1 receptor, the receptor for LTN (Cairns et al. 2001). Another approach to use LTN as an enhancer of immune responses was with the manipulation of dendritic cells (DCs) for the expression of this chemokine. The administration of the DCs expressing LTN caused a potent adjuvant effect for peptide delivery causing specific antitumor immunity in mice (Cao et al. 1998).

Our goal was to create an innocuous and safe vector to produce, but mostly to deliver, LTN in vivo. In recent years, the lactic-acid bacterium *L. lactis* has been used as an efficient vector to express heterologous proteins of medical and therapeutic interest (Wells and Mercenier 2008). The intranasal immunization with strains of this food-grade bacterium has resulted in the generation of specific humoral and cytotoxic immune responses against infectious and chronic diseases (Braat et al. 2006; Hanniffy et al. 2007; Wells and Mercenier 2008). Because of these promising results using this microorganism, we genetically manipulated a strain of *L. lactis* to express and secrete human lymphotactin (huLTN). Here we demonstrate how this *L. lactis*-secreted LTN remains biologically active and able to chemoattract T lymphocytes by chemotaxis. This is the first report that demonstrates the biological activity of huLTN secreted by a food-grade lactic acid bacterium.

Materials and methods

Bacterial strains and growth conditions

Lactococcus lactis NZ9000 (Kuipers et al. 1997) was grown aerobically in M17 medium (DIFCO) supplemented with 1% glucose (GM17) at 30°C without agitation. *Escherichia coli* DH5α was grown in Luria–Bertani (LB) medium at 37°C with aeration and vigorous shaking. Unless otherwise indicated, construction were first established in *E. coli* and then transferred to *L. lactis* by electrotransformation (Holo and Nes 1989). Clones were selected by addition of 100 μg ampicillin/ml or 10 μg chloramphenicol/ml for *E. coli* and 10 μg chloramphenicol/ml for *L. lactis*. PCR was done using Vent DNA polymerase (New England Biolabs) according to the manufacturer's instructions using a programmable thermal controller.

Harvest and culture of macrophages

Human macrophages were isolated from freshly drawn blood by using Ficoll–Hypaque (Histopaque-1077, Sigma). The layer of mononuclear cells was recovered, washed with PBS, and cultured for 3 h in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and then incubated at 37°C in a 5% CO_2. Adherent cells (macrophages) were recovered by removing the culture medium. Macrophages were seeded into 6-well plates (Costar) at 5×10^6 cells/ml in complete RPMI-1640 medium (10% v/v heat-inactivated FBS, 1% penicillin–streptomycin solution, and 1% HEPES buffer) stimulated with 20 mg lipopolysaccharides/ml (LPS B from *E. coli* 026:B6, Sigma) and incubated at 37°C in a 5% CO_2 for 5 h.

Human cDNA preparation

The total RNA from 5×10^6 cells of LPS-stimulated human macrophages was isolated using TRizol reagent (Gibco) according to the manufacturer's instructions. The concentration and integrity of RNA was determined by measuring absorbance at 260 nm and analyzed by formaldehyde–agarose gel electrophoresis. The first strand cDNA was synthesized from 1 μg total RNA by SuperscriptTM II reverse transcriptase (Gibco) and oligo (dT) 12–18 primer.

Protein extraction from *L. lactis*

Cell and cell-free medium fractions were prepared separately. Samples were processed from 1.35 ml of cultures of *L. lactis*. Cell pellets were obtained by centrifugation at 21000*g* at 4°C for 5 min. Cell pellets were then resuspended in 100 μl lysis buffer containing 25% (w/v) sucrose, 1 mM EDTA, 50 mM Tris/HCl, pH 8.0, lysozyme (10 mg/ml) complemented with 1 mM PMSF to avoid proteolysis and 10 mM DTT and incubated at 37°C for 1 h. Samples were kept at −20°C before loading onto gel.

The cell-free medium samples were treated with 1 mM PMSF and 10 mM DTT to avoid proteolysis. Proteins were precipitated adding 150 μl of 100% trichloroacetic acid (TCA) to 1.35 ml cell-free medium (TCA at 10% w/v) and incubated on ice for 10 min followed by centrifugation at 21000*g* at

4°C for 15 min. The pellet was resuspended in 50 µl of 50 mM NaOH and 50 µl of 2× SDS-PAGE loading buffer (100 mM Tris/HCl, pH 6.8, 200 mM DTT, 4% v/v SDS, 0.1% Bromophenol Blue, and 10% v/v glycerol).

SDS-PAGE and western blotting

Samples were electrophoresed in 12% polyacrylamide gels. Immunodetection was done using polyclonal anti-lymphotactin (Santa Cruz) as a primary antibody and protein-G horseradish-peroxidase conjugate (BioRad). SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used as recommended by the suppliers.

Lymphocyte culture

Human peripheral blood lymphocytes were isolated from donor whole blood by centrifugation on Ficoll–Hypaque (Histopaque-1077 Sigma) according to manufacturer's instructions. After washing three times with RPMI 1640 medium (Gibco), the cells were cultured in RPMI 1640 medium supplemented with 10% human serum (Sigma) and 200 U human interleukin 2/ml (Santa Cruz Biotechnologies) at 37°C in a 5% CO_2 for 12 days. Cell density was kept between 1 and 3 × 10^6 cells/ml.

Chemotaxis assay and flow cytometry

Cell migration was measured in 5 µm diameter pore-size cellulose-nitrate membranes (Whatman) using a Boyden chemotaxis chamber. Cell-free medium from wild type and recombinant *L. lactis* cultures were sterilized by filtration through a 0.22 µm filter (Millex, Millipore) and loaded into the lower compartment of Boyden chamber for chemotaxis. Lymphocytes (10^5 cells) in RPMI 1640 medium were loaded into the upper compartment. Chemotaxis was allowed to occur for 1 h at 37°C. Migrated cells were counted by a fluorescence-activated cell-sorting (FACS) flow cytometer. The aqueous phase from the lower compartment of chambers was gently recovered and centrifuged. The resulting cell pellet was suspended in PBS and incubated for 10 min with human anti-CD3 FITC (BD Biosciences Pharmingen, San Diego, USA), washed with PBS, and counted with an FACS sorting cytometer. All chemotaxis assays were done in triplicate. Complement-activated serum was prepared from fresh human serum by addition of 25 mg zymosan (Sigma) per ml serum and incubated at 37°C for 1 h. Statistical analysis was done using the Tukey test.

Results and discussion

Construction of an inducible system for LTN secretion by *L. lactis*

Lymphotactin is a chemokine produced by leukocytes upon stimulation. To obtain a DNA sequence coding for huLTN, we first constructed a cDNA from human macrophages stimulated with LPS. The DNA sequence coding for the mature moiety was PCR amplified from this human cDNA with the following oligonucleotides: LptnHP9rw: (5′-T**ATGCAT**CAGT AGGGAGTGAAATCTCA-3′) and reverse primer (LptnHLBfw: 5′-CTAGCCAGTCAGGGTCACAGC TGT-3′), which generated a *Nsi*I restriction site (indicated in bold) to manipulate the fragment for future cloning. The 298 bp amplicon was directly cloned into the pCR2.1 vector (Invitrogen) and named pCR2.1-huLTN.

For the generation of the plasmid responsible for regulation of expression and secretion of LTN in *L. lactis*, the plasmid pCR2.1-huLTN was digested with *Nsi*I–*Eco*RI releasing the fragment coding for LTN and subcloned into the pSEC-E7 vector digested with the same enzymes. The final construct was named pSEC-huLTN. This strategy permitted the fusion in-frame of the DNA sequence coding for LTN with the signal peptide from the *usp45* (SP_{Usp45}), the most secreted protein of *L. lactis* (van Asseldonk et al. 1990), and placed under the regulation of the well-characterized nisin-inducible promoter (P*nisA*) (Fig. 1).

Finally our construction was introduced into the *L. lactis* strain NZ9000 carrying the regulatory genes *nisR* and *nisK* (de Ruyter et al. 1996) by electrotransformation as previously described (Holo and Nes 1989). Recombinant strains carrying the final construct were selected by resistance to chloramphenicol and named NZSEC:huLTN. All plasmids were endonuclease-characterized and sequenced for DNA integrity.

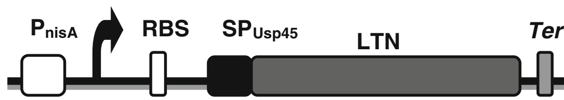

Fig. 1 Schematic representation of LTN expression system for production and secretion by *L. lactis*. The scheme represents the regulatory system to express huLTN by *L. lactis*, controlled by inducible promoter P_{nis} (P_{nis}), and using the ribosome binding site (RBS) and signal peptide of the of Usp45 protein (SP_{usp45}). The final gray bar (from left to right) represents a rho-independent *trpA* transcription terminator. (Not to scale)

Secretion of LTN by *L. lactis* is efficient

To evaluate the functionality and efficiency of the system for secretion of LTN, three transformants (NZSEC:huLTN) were induced with nisin for just 1 h to allow LTN expression and then analyzed by Western blot using polyclonal anti-lymphotactin antibodies. Protein extractions were made separately for cell and cell-free medium fractions.

Western blot analysis of samples from the cell-free medium from the induced cultures revealed a single band of 10 kDa, the expected molecular mass of LTN, indicating that that protein corresponded to LTN secreted by *L. lactis* (Fig. 2). No degradation or incomplete forms of the LTN were detected, showing an efficient secretion of the chemokine by *L. lactis.* To evaluate the functionality of the inducible system and to determine if the system remained completely shut-down in the absence of the inducer (nisin), we analyzed samples from non-induced cultures of these same recombinant strains. No signal was detected in any of the non-induced samples, showing how the expression of the LTN is strictly regulated by the nisin-inducible system, where in the absence of nisin, the expression of LTN is completely turned off (Fig. 2). No signal was detected in either of the cell fractions from induced and non-induced cultures (data not shown). Samples from the cell-free medium from wild-type *L. lactis* were included as controls. As expected, no signal for LTN was detected (Fig. 2).

Secreted amount of LTN by *L. lactis*

To estimate the relative amount of LTN secreted by *L. lactis*, cultures from recombinant NZSEC:huLTN at an $OD_{600\ nm}$ of 0.6 were induced for 1 h followed by protein extraction from the cell-free medium as before. Serial dilutions were compared against fixed amounts of human recombinant lymphotactin (RnD Systems) by western blot analysis. The undiluted extract in Fig. 3, which has the highest intensity, corresponds to protein precipitated from 675 µl cell-free medium from an induced culture of NZSEC:huLTN. Analysis of band intensities with computer software (Phoretix 1D, Nonlinear dynamics) indicates that our recombinant *L. lactis* secretes at least 590 ng of LTN per ml of culture. This result demonstrates how this strain of *L. lactis* is able to secrete more than 500 ng huLTN protein in just 1 h/ml of culture.

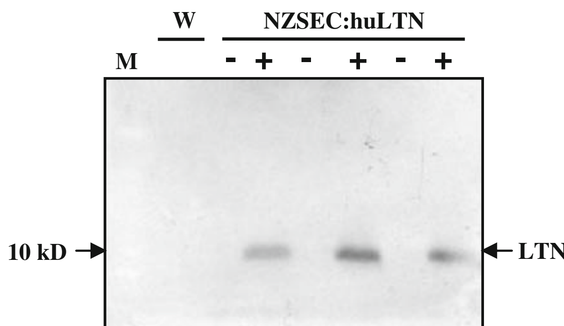

Fig. 2 Efficient secretion of huLTN by *L. lactis*. Three different clones from recombinant *L. lactis* (NZSEC:huLTN) were analyzed by Western blot for secretion of huLTN. Cell-free medium samples of induced (+) and non-induced (−) cultures. WT, cell-free medium from wild type strain. M, protein molecular marker

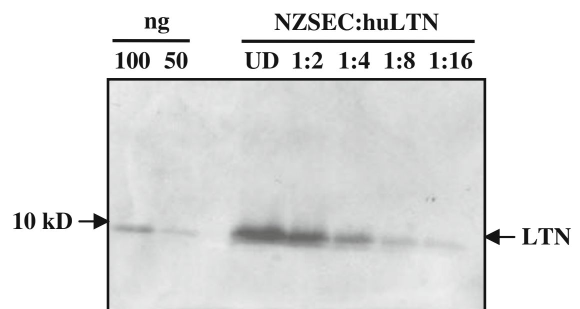

Fig. 3 Semiquantitative analysis of LTN secreted by *L. lactis*. Cultures of recombinant *L. lactis* (NZSEC:huLTN) grown were induced with 10 ng/ml of nisin for 1 h. The cell-free medium samples were processed and serial dilutions from the total extract from 1:2 to 1:16 were prepared. Samples and two fixed amounts of pure recombinant LTN (50 and 100 ng) were analyzed by Western Blot using a specific anti-lymphotactin antibody. Undiluted sample (UD) represents the amount of LTN in 675 µl from a *L. lactis* culture induced for 1 h

Biological activity of *L. lactis*-secreted LTN

Lymphotactin is a glycosylated chemokine able to direct migration of T lymphocytes and NK cells (Dorner et al. 1997; Lillard et al. 1999). Because we achieved the expression of this LTN in *L. lactis*, our next step was to determine the biological activity of this *L. lactis*-secreted chemokine. We measured the ability to chemoattract human T lymphocytes by the *L. lactis*-secreted LTN in a chemotaxis assay. For this we added 10^5 cells of activated T lymphocytes to the upper compartment of the chemotaxis chamber and cell-free medium from cultures of *L. lactis* to the bottom compartment. Chemotaxis was allowed to occur for 1 h at 37°C and then the attracted cells to the bottom of the chamber were recovered. After centrifugation, the cells were washed with PBS and incubated with human anti-CD3+ and counted with a FACS flow cytometer. The cell counts showed that the LTN present in medium from induced cultures of recombinant strain NZSEC:huLTN induced chemoattraction of more than 15,000 T cells (Fig. 4), indicating the ability of this chemokine secreted by a gram positive bacterium to chemoattract human T lymphocytes. The cell-free medium from a culture of wild-type *L. lactis* and PBS were used as negative controls and did not attract significant amounts of cells. Complement-activated serum was used as a positive control (Fig. 4).

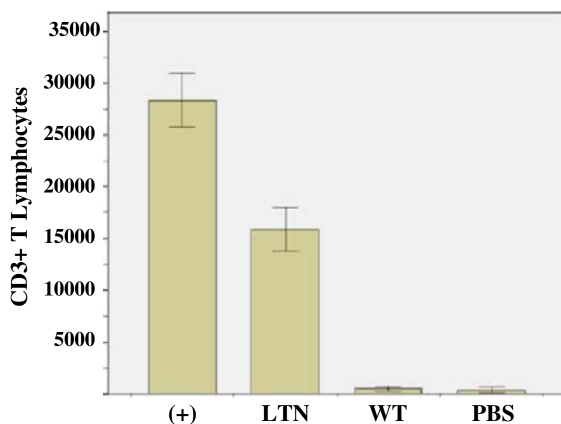

Fig. 4 Secreted LTN by *L. lactis* is biologically active. Chemotactic activity of LTN secreted by *L. lactis* was evaluated by chemotaxis of activated human T lymphocytes in Boyden chambers. After chemotaxis, attracted cells were counted by FACS analysis using an anti-CD3+ antibody. Complement activated human serum was used as a positive control and as negative controls cell-free medium from WT strain (WT) and PBS

These results demonstrate for the first time the expression and secretion of a C subfamily chemokine of human origin by *L. lactis*, biologically active, able to cause chemoattraction of human T lymphocytes (Fig. 4).

Antecedents have demonstrated the use of *L. lactis* as a biological vector to deliver antigens and cytokines at mucosal surfaces to cause humoral and cytotoxic immune responses (Wells and Mercenier 2008). Here we report a novel strain of *L. lactis* able to secrete a biologically active antitumor chemokine. This food-grade bacterium represents a feasible well-tolerated vector intended to be used in live organisms to deliver huLTN at a mucosal level.

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