

BASIC–ALIMENTARY TRACT

Induction of Ovalbumin-Specific Tolerance by Oral Administration of *Lactococcus lactis* Secreting Ovalbumin

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Background & Aims: Obtaining antigen-specific immune suppression is an important goal in developing treatments of autoimmune, inflammatory, and allergic gastrointestinal diseases. Oral tolerance is a powerful means for inducing tolerance to a particular antigen, but implementing this strategy in humans has been difficult. Active delivery of recombinant autoantigens or allergens at the intestinal mucosa by genetically modified *Lactococcus lactis* (*L. lactis*) provides a novel therapeutic approach for inducing tolerance. **Methods:** We engineered the food grade bacterium *L. lactis* to secrete ovalbumin (OVA) and evaluated its ability to induce OVA-specific tolerance in OVA T-cell receptor (TCR) transgenic mice (DO11.10). Tolerance induction was assessed by analysis of delayed-type hypersensitivity responses, measurement of cytokines and OVA-specific proliferation, phenotypic analysis, and adoptive transfer experiments. **Results:** Intragastric administration of OVA-secreting *L. lactis* led to active delivery of OVA at the mucosa and suppression of local and systemic OVA-specific T-cell responses in DO11.10 mice. This suppression was mediated by induction of CD4⁺CD25[−] regulatory T cells that function through a transforming growth factor β -dependent mechanism. Restimulation of splenocytes and gut-associated lymph node tissue from these mice resulted in a significant OVA-specific decrease in interferon γ and a significant increase in interleukin-10 production. Furthermore, Foxp3 and CTLA-4 were significantly up-regulated in the CD4⁺CD25[−] population. **Conclusions:** Mucosal antigen delivery by oral administration of genetically engineered *L. lactis* leads to antigen-specific tolerance. This approach can be used to develop effective therapeutics for systemic and intestinal immune-mediated inflammatory diseases.

The mucosal immune system maintains an equilibrium between tolerance toward commensals and harmless agents (eg, food antigens) on the one hand and active immunity toward pathogenic agents on the other.¹ Disturbance of this balance is an important pathogenic mechanism in many different autoimmune, allergic, and inflammatory gastrointestinal diseases. Induction of antigen-specific oral tolerance (OT) is an attractive therapeutic approach because it generally lacks toxicity, can be easily administered over time, and avoids adverse effects associated with generalized immune suppressive intervention. Oral administration of (auto)antigens or allergens can effectively prevent the induction of autoimmune and allergic diseases in animal models, but several clinical attempts to induce OT for therapeutic purposes have failed.^{2–4} These failures may be related to the source, the purity, and the amount of (auto)antigen needed or to the way the antigen is presented to the mucosal immune system. Experimental data indicate that heterogeneous antigen mixtures are less effective inducers of OT than single purified antigens and that the antigen dose is critical. High doses of antigen can lead to clonal deletion or anergy of the T cells recognizing the antigen, whereas low doses can induce active suppression, eg, by inducing antigen-specific regulatory T cells secreting suppressive cytokines.^{5–7} When anergy or clonal deletion is desired,⁸ the antigen has to be known. However, if multiple pathogenic antigens are implicated, or when the causal antigen is unknown, therapeutic effects can be induced by generating “bystander” regulatory T cells.^{2,9,10} Different types of regulatory T cells can be induced or expanded by mucosal antigens, including CD4⁺CD25⁺, CD4⁺CD25[−],

Abbreviations used in this paper: APC, antigen presenting cell; BM, bone marrow; DC, dendritic cells; DTH, delayed type hypersensitivity; GALT, gut-associated lymphoid tissue; IFN, interferon; IL, interleukin; LL, *Lactococcus lactis*; LL-OVA, OVA-secreting *L. lactis*; OT, oral tolerance; OVA, ovalbumin; TCR, T-cell receptor; Treg, regulatory T cell.

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and CD8⁺ regulatory T cells through a transforming growth factor (TGF)- β and/or interleukin (IL)-10-dependent mechanism.²

The *Lactococcus lactis* (*L. lactis*)-mediated delivery system obviates the need for large scale purification of human autoantigens or allergens and enables delivery of antigens to the intestinal mucosa. *L. lactis* is a nonpathogenic, noninvasive, noncolonizing gram-positive bacterium, and, according to the US Food and Drug Administration, it is generally regarded as safe. Previously genetically modified *L. lactis* strains have been produced for synthesis and delivery of immunomodulatory proteins at the intestinal mucosa, and an adequate biologic containment system has been established.^{11–13} A biologically contained *L. lactis* strain secreting human IL-10 was used in a phase I, open label clinical trial on Crohn's disease patients. This trial demonstrated that treatment of humans with viable *L. lactis* secreting IL-10 is clinically and biologically safe and gave indications of its clinical efficacy.¹⁴ The use of genetically modified *L. lactis* for intestinal delivery of proteins in humans warrants further investigation.

In the present study, we evaluated the efficacy of *L. lactis* as a vehicle for intestinal delivery of antigens for the induction of antigen-specific peripheral tolerance. We fed ovalbumin (OVA)-immunized DO11.10 mice, which bear transgenic OVA-specific CD4⁺ T-cell receptors, with OVA-secreting *L. lactis* (LL-OVA). Our data indicate that LL-OVA leads to OVA-specific tolerance by inducing CD4⁺CD25⁺ regulatory T cells that function through a TGF- β -dependent mechanism. These cells can transfer tolerance to OVA-immunized wild-type Balb/c recipients. This intestinal delivery system is more effective than oral administration of purified antigen. Thus, live, genetically modified *L. lactis* holds promise as a tool for efficient induction of antigen-specific peripheral tolerance in humans suffering from antigen-driven immune diseases.

Materials and Methods

Bacteria and Media

The *Lactococcus lactis* MG1363 (LL) strain¹⁵ was genetically modified and used throughout this study. Bacteria were cultured in GM17E medium consisting of M17 broth (Difco Laboratories, Detroit, MI) supplemented with 0.5% glucose and 5 μ g/mL erythromycin (Abbott B.V., Hoofddorp, The Netherlands). Stock suspensions of the *Lactococcus lactis* MG1363 strains were stored at -20°C in 50% glycerol in GM17E medium. Stock suspensions were diluted 200-fold in GM17E medium and incubated at 30°C overnight. Within 16 hours, they reached a saturation density of 2×10^9 colony-forming units (CFU) per mL. Bacteria were harvested by centrifugation and resuspended in BM9 medium at 2×10^{10} bacteria/mL. Each mouse received 100 μ L of this suspension daily through an intragastric catheter.

Plasmids

The messenger RNA (mRNA) sequence encoding *Galus gallus* ovalbumin was retrieved from Genbank (accession number AY223553) and from published data.¹⁶ Total RNA was isolated from chicken uterus and complementary DNA (cDNA) was synthesized using 2 μ g total RNA, 2 μ mol/L oligo dT primers (Promega Corporation Benelux, Leiden, The Netherlands), 0.01 mmol/L DTT (Sigma-Aldrich, Zwijndrecht, The Netherlands), 0.5 mmol/L dNTP (Invitrogen, Merelbeke, Belgium), 20 U Rnasin (Promega Incorporation Benelux), and 100 U superscript II reverse transcriptase (Invitrogen) in a volume of 25 μ L. An OVA cDNA fragment was amplified by polymerase chain reaction (PCR) using the following primers: forward 5'-GGCTCCATCGGTGCAGCAAGCATGGAATT-3' and reverse 5'-ACTAGTTAAGGGGAAACACATCTGCCAAAGAA GAGAA-3'. Reaction conditions were 94°C for 2 minutes followed by 30 cycles at 94°C for 45 seconds, 62°C for 30 seconds, and 72°C for 90 seconds. The amplified fragment was fused to the Usp45 secretion signal¹⁷ of the erythromycin resistant pT1NX vector, downstream of the lactococcal P1 promoter.¹⁸ MG1363 strains transformed with plasmids carrying OVA cDNA were designated *L. lactis* secreting OVA (LL-OVA). The *L. lactis*-pT1NX, which is MG1363 containing the empty vector pT1NX, served as control (LL-pT1NX).¹⁵

Mice

Seven-week-old female Balb/c mice were obtained from Charles River Laboratories (Calco, Italy). OVA-specific T-cell receptor transgenic mice (DO11.10) on a BALB/c background were kindly provided by Dr J. Samson (Vrije Universiteit, Amsterdam, The Netherlands) and bred at the Academic Medical Center, Amsterdam. DO11.10 mice were used at the age of 8–10 weeks. All mice were housed in a conventional animal facility under routine laboratory conditions. All experiments were approved by the Animal Experiments Committee of the Academic Medical Center.

Antigen and Antibodies

Intact, lipopolysaccharide (LPS)-free OVA grade V protein was used as antigen in all experiments (Sigma Aldrich). Anti-CD4, anti-CD25, and anti-CTLA-4 antibodies were purchased from BD-Pharmingen (Woerden, The Netherlands), and anti-Foxp3 antibodies were obtained from eBiosciences (San Diego, CA).

In Vitro and In Vivo Quantification of OVA Secreted by *L. lactis*

Female BALB/c mice were fed 10 serial inoculates of 2×10^9 CFU of LL-OVA in 100 μ L BM9 medium, or 100 μ L BM9 medium alone (control), once every 30 minutes. One hour after the final inoculation, intestinal segments were homogenized in phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS) for analyses. OVA production was quantified by enzyme-linked immunosorbent assay (ELISA) and live *L. lactis* bacterial

count by plating 10-fold dilutions of the homogenates on GM17E agar plates containing 5 $\mu\text{g/mL}$ erythromycin. OVA secreted in vivo was quantified by sampling the proximal and distal small intestines, the cecums, and the colons in 2 different ways: (1) samples of the entire intestine, including luminal content, and (2) extensively washed intestinal tissue samples for measurement of mucosal OVA concentrations. Samples were homogenized, and OVA was quantified by ELISA. OVA was captured from the saturated culture supernatant by immobilized polyclonal rabbit anti-OVA antibody (2 $\mu\text{g/mL}$; Research Diagnostics Inc), quantified by anti-OVA biotin-conjugated polyclonal rabbit antibody (2.5 $\mu\text{g/mL}$; Research Diagnostics Inc), and stained with horseradish peroxidase-conjugated streptavidin (1/1000; BD Pharmingen, San Diego, CA) followed by 3,3', 5,5' tetramethylbenzidine substrate (BD Pharmingen).

Oral Feeding and Delayed-Type Hypersensitivity

DO11.10 mice were immunized by subcutaneous (SC) injection of 100 μg OVA in 50 μL of a 1:1 mixture of Complete Freund's adjuvant (Difco, BD, Alphen aan de Rijn, The Netherlands) and saline solution at the base of the tail on the first day.¹⁹ Mice were fed purified OVA dissolved in 100 μL BM9 as follows: 200 mg on day 8, 1 mg on days 1–5, and 1 μg on days 1–5 and 8–12. LL-OVA or LL-pT1NX was administered on days 1–5 and 8–12. Control mice received only BM9. Antigen or bacterial suspensions were introduced into the stomach using an 18-gauge stainless animal feeding needle. Eleven days after sensitization, antigen-specific, delayed-type hypersensitivity (DTH) responses were assessed by injection of OVA. Twenty-four hours later, DTH measurements were performed, or spleen and lymph nodes were harvested and the cells assessed for OVA-specific proliferation and cytokine production. For measurement of antigen-specific DTH responses, mice were challenged with 10 μg OVA in 10 μL saline in the auricle of one ear and 10 μL saline in the other. Ear swelling, defined as the increase in ear thickness because of challenge, was measured in a blinded fashion 24 hours after challenge using a micrometer (Mitutoyo, Tokyo, Japan). DTH responses were expressed as the difference in swelling between the OVA-injected and the saline-injected ears (swelling because of OVA minus swelling because of saline).

Cell Cultures and Proliferation and Cytokine Assays

Single cell suspensions of spleen and lymph nodes were prepared by passing the cells through 70- μm cell strainers (Becton/Dickinson Labware). Erythrocytes in the spleen cell suspensions were lysed by incubation with red cell lysis buffer. For IL-10 measurements, 1×10^5 lymphocytes isolated 1 day after DTH challenge were incubated in triplicate 96-well plates (Costar, Cambridge, MA) in 200 μL complete medium. They were either left

unstimulated or were stimulated in the presence of plate-bound anti-CD3 (1:30 concentration; clone 145.2C11, a gift from Dr R. Mebius) and soluble anti-CD28 (1:1000 concentration; BD Biosciences) for 48 hours or with 500 $\mu\text{g/mL}$ OVA for 40 hours. CD4⁺ T cells and CD4⁺CD25[−] T cells were enriched using CD4⁺ T-cell isolation kit or CD4⁺CD25⁺ regulatory T-cell isolation kit and midi-MACS columns (all materials from Miltenyi Biotec, Germany).

A T-cell proliferation assay was performed using DO11.10 CD4⁺ T cells and bone marrow (BM)-derived CD11c⁺ dendritic cells (DC) cultured with LL-OVA or LL-pT1NX. BM-derived DCs were generated as described²⁰ and positively selected using anti-CD11c magnetic microbeads and MACS columns (Miltenyi Biotec, Utrecht, The Netherlands) according to manufacturer's instructions. The CD11c⁺ cells were plated at 2×10^6 per well (24-well; Costar) with 4×10^5 CFU *L. lactis* or with 1 μg or 1 mg OVA per milliliter in RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamax, 0.1 mmol/L nonessential amino acids, 0.4 mol/L sodium pyruvate, 25 mmol/L HEPES (all 4 from Invitrogen), 50 $\mu\text{mol/L}$ β -mercaptoethanol (2-ME), and 5 $\mu\text{g/mL}$ erythromycin (Abbott). After 4-hour incubation, the bacteria were killed by adding 75 $\mu\text{g/mL}$ gentamycin. Twenty hours later, cells were harvested. Two $\times 10^5$ CD4⁺ T cells were then cultured with the harvested DCs at ratios of 1/0.3, 1/0.11, 1/0.03, and 1/0.01, respectively. The cultures were grown in 96-well U-bottom plates (Becton Dickinson, Alphen aan de Rijn, The Netherlands) in a total volume of 200 μL complete medium consisting of RPMI-1640 with 10% FCS, 2 mmol/L L-glutamax (Invitrogen), 0.1 mmol/L nonessential amino acids, 0.4 mol/L sodium pyruvate, 25 mmol/L HEPES, 50 $\mu\text{mol/L}$ 2-ME, 10 U/mL penicillin (Invitrogen), and 10 $\mu\text{g/mL}$ streptomycin (Invitrogen).

To assay proliferation of total splenocyte populations, 2×10^5 cells were cultured in 96-well U-bottom plates in a total volume of 200 μL complete medium either alone or with OVA and either with or without anti-IL-10 neutralizing monoclonal antibody (1 $\mu\text{g/mL}$, clone JES052A5; R&D Systems, Abingdon, United Kingdom). OVA was added at concentrations ranging from 1.2 to 100 $\mu\text{g/mL}$. The anti-IL-10 antibody was added at 1.0, 0.1, and 0.01 $\mu\text{g/mL}$. To assay proliferation of CD4⁺ T cells and CD4⁺CD25[−] T-cell populations, 0.2×10^5 CD4⁺ T cells or CD4⁺CD25[−] T cells were cultured in 96-well U-bottom plates with 1×10^5 irradiated CD4[−] cells, acting as antigen presenting cells (APC), and OVA (0 or 100 $\mu\text{g/mL}$) in a total volume of 200 μL complete medium either with or without TGF- β neutralizing monoclonal antibody (1 $\mu\text{g/mL}$, clone 1D11; R&D Systems). Cells were cultured for 90 hours at 37°C in 5% CO₂ in a humidified incubator. For proliferation assays, 1 $\mu\text{Ci/well}$ [³H]-thymidine was added for the last 18 hours of culture, DNA was harvested on glass fiber filter mats

(Perkin Elmer, Boston, MA), and DNA-bound radioactivity was measured on a scintillation counter (Perkin Elmer). For cytokine measurements, supernatants of the cell cultures used in the different proliferation assays were collected after 72 hours of culture and frozen at -20°C . Cytokine production was quantified using the Mouse Inflammation Cytometric Bead Assay (BD Biosciences, Mountain View, CA).

Flow Cytometry Analysis

On day 12, spleens of mice treated with BM9, $1\ \mu\text{g}$ OVA, LL-pT1NX, or LL-OVA were isolated and enriched for $\text{CD4}^{+}\text{CD25}^{-}$ T cells using CD4^{+} T-cell isolation kit or $\text{CD4}^{+}\text{CD25}^{+}$ regulatory T-cell isolation kit, as described above. To determine the regulatory phenotype of the $\text{CD4}^{+}\text{CD25}^{-}$ T-cell population, cells were stained for Foxp3 and CTLA-4 and analyzed by flow cytometry (FACScan; Becton Dickinson, Woerden, The Netherlands).

Adoptive Transfer Experiments

Wild-type Balb/c donor mice were immunized by SC injection with $100\ \mu\text{g}$ OVA in $50\ \mu\text{L}$ of a 1:1 mixture of CFA (Difco; Becton Dickinson, Alphen aan de Rijn, The Netherlands) and saline solution at the base of the tail on days 0 and 1. On day 7, CD25 subsets isolated

from spleens of DO11.10 mice fed with LL-OVA (as previously described) were adoptively transferred in the sensitized Balb/c mice. To obtain the CD25 subsets, spleen cells were stained using the $\text{CD4}^{+}\text{CD25}^{+}$ regulatory T-cell isolation kit (Miltenyi Biotec) and sorted by MACS cell sorting. The $\text{CD4}^{+}\text{CD25}^{+}$ and $\text{CD4}^{+}\text{CD25}^{-}$ populations were sorted again using a FACS diva (BD, Becton Dickinson, Erembodegem, Belgium), which separated the positive population into $\text{CD25}^{\text{high}}$ and $\text{CD25}^{\text{intermediate}}$ subpopulations. Immediately afterwards, 5×10^4 $\text{CD25}^{\text{high}}$, $\text{CD25}^{\text{intermediate}}$, or CD25^{-} cells in $200\ \mu\text{L}$ PBS were adoptively transferred by intravenous (IV) injection into the OVA-immunized Balb/c mice. These mice were then challenged on day 12 with $10\ \mu\text{g}$ OVA in $10\ \mu\text{L}$ saline in the auricle of one ear and $10\ \mu\text{L}$ saline in the other ear, and DTH responses were measured 24 hours later, as described previously.

Statistical Analysis

Results from cytokine measurements are expressed as means $\pm$ SEM. Significance of differences between groups in ear thickness and cytokine measurements were tested using 1-way ANOVA followed by Student *t* test. Significance of OVA-specific proliferation was evaluated using a general linear model with repeated

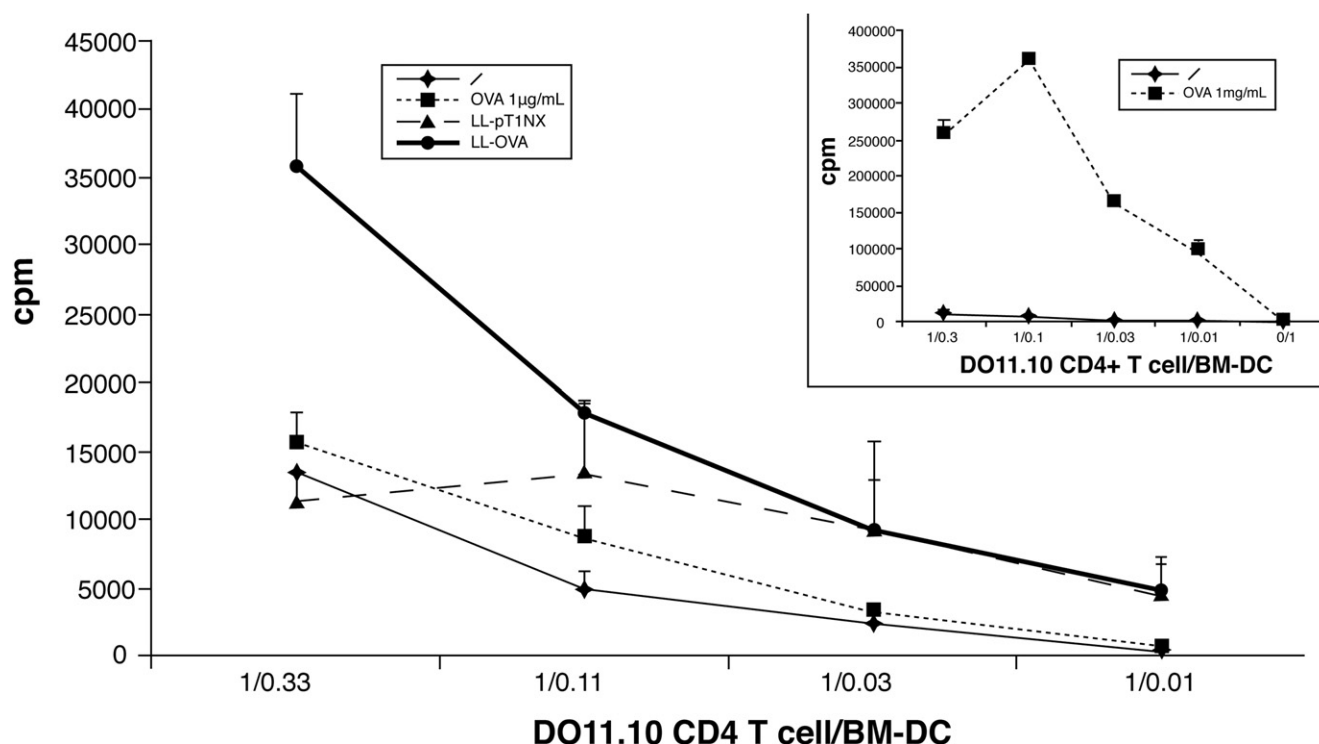


Figure 1. LL-OVA induces APC-mediated T-cell proliferation. *L. lactis*-derived OVA is bioactive because it induces proliferation of DO11.10 CD4^{+} T cells after preincubation of BM-DC with LL-OVA. BM-derived DC were generated as described in the Materials and Methods section. Two $\times 10^6$ BM-DC were cultivated with 4×10^5 CFU LL-OVA or LL-pT1NX, with $1\ \mu\text{g}$ or $1\ \text{mg}$ OVA per mL or with no additive ($\emptyset$). After 4 hours, bacteria were killed with gentamycin. Twenty hours later, BM-DC were harvested, and their proliferation was assayed using DO11.10 CD4^{+} T cells. Two $\times 10^5$ DO11.10 CD4^{+} T cells were incubated with 6.67×10^4 , 2.22×10^4 , 7.41×10^3 , and 2.47×10^3 BM-DC, corresponding to 1/0.33, 1/0.11, and 1/0.01, respectively, CD4^{+} T cells/BM-DC. Given the considerable higher proliferation after stimulation with $1\ \text{mg/mL}$ OVA, this proliferation is shown in a separate graph.

measurements. For both tests, statistical significance is indicated as * $P < .05$ or ** $P < .01$.

Results

LL-OVA Induces APC-Mediated T-Cell Proliferation In Vitro

A *L. lactis* strain that secretes chicken OVA, designated LL-OVA, was constructed. In vitro synthesis of OVA was evaluated by ELISA. OVA secretion did not alter the growth rate of *L. lactis*, and, after 16 hours of growth, OVA was detected in the culture supernatant at a concentration of 7 ± 2 ng/mL. No intracellular OVA could be detected, demonstrating efficient secretion of OVA.

LL-OVA were able to induce BM-DC maturation (data not shown) and proliferation of DO11.10 CD4⁺ T cells that were cocultured with BM-DC. Less than 0.5 ng/mL of OVA was produced by LL-OVA during a 4-hour coculture of BM-DC with LL-OVA. Purified OVA at 1 mg/mL induced significantly more proliferation than LL-OVA, but, at 1 μ g/mL, it failed to induce any proliferation (Figure 1). This clearly demonstrates that *L. lactis*-derived OVA is bioactive and suggests that the LL-OVA-induced T-cell proliferation is mediated by APC.

Active Intestinal Delivery of OVA by L. lactis

To quantify the in vivo secretion of OVA, small intestines, cecums, and colons were obtained 1 hour after the final inoculation. CFU numbers and OVA concentrations were determined within the entire intestine, including its luminal content as well as within the intestinal tissue, ie, the mucus and mucosa. No live *L. lactis* bacteria were found in the proximal small intestine, whereas, in samples of luminal content from the distal small intestine, the cecum, and the colon, CFU averaged 7.25×10^9 , 2.49×10^9 , and 3.49×10^9 , respectively. Relatively few viable *L. lactis* were found in contact with mucus or within the mucosa of the distal small intestine (4.16×10^6 CFU), cecum (7.03×10^7 CFU), and colon (1.41×10^6 CFU) (Table 1). The largest amount of OVA derived from LL-OVA was detected in the colon (48 ng per colon), but only a small fraction of the colon-delivered OVA (3 ng per colon) was found in the intestinal tissue. A smaller amount of OVA was found in the distal part of the small intestine, but this was localized entirely in the mucus and/or mucosa. No OVA was detected in the proximal small intestine (Figure 2). This indicates that *L. lactis* actively produced OVA in vivo, which was mostly recovered from the mucus and/or mucosa of the small bowel and in the lumen of the large intestine.

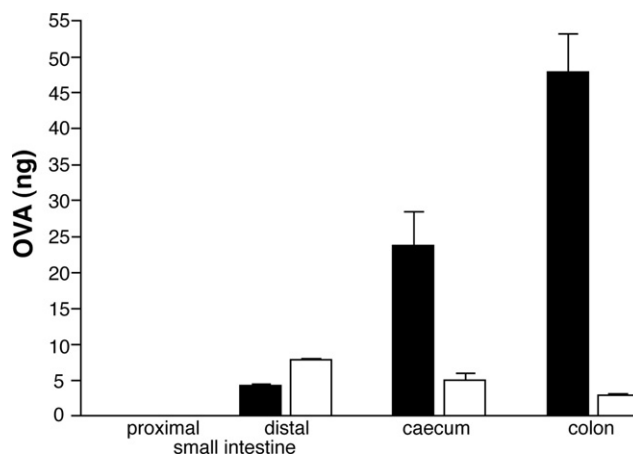


Figure 2. OVA is actively produced by LL-OVA in the intestine and is delivered to the small and large intestines. Balb/c mice ($n = 4$) received 10 serial inocula of 2×10^9 CFU of LL-OVA in 100 μ L suspension at intervals of 30 minutes. One hour after the final inoculation, live bacteria (Table 1) and OVA (Figure 2) were quantified in the entire small intestines, cecums, and colons, including their luminal contents (solid bar), and in the intestinal tissue (open bar), as described in the Materials and Methods section. Detection limit of the ELISA = 1.5 ng. Results are representative of 2 individual experiments.

Administration of LL-OVA Significantly Suppresses OVA-Induced DTH

OVA-specific DTH reactions in the ear are accurately reflected in ear swelling.^{21,22} Using an intragastric catheter, OVA-immunized mice were fed BM9 (as a negative control), 200 mg OVA on day 8, 1 mg OVA on days 1–5, 1 μ g OVA on days 1–5 and 8–12, or LL-pT1NX (as vector control) or LL-OVA on days 1–5 and 8–12. On day 11, mouse ears, after measurement of thickness, were injected with 10 μ g OVA. Ear thickness was measured again after 24 hours, and swelling was measured as the increase in ear thickness. Control mice were significantly sensitized to OVA, as evidenced by ear swelling, but daily intragastric administration of LL-OVA significantly reduced this swelling (15×10^{-2} mm vs 1×10^{-2} mm, respectively, $P = .0152$) (Figure 3). Ear swelling was also somewhat reduced in LL-pT1NX-treated mice compared with controls (BM9) (6.5×10^{-2} mm vs 15×10^{-2} mm, respectively). Remarkably, mice that were fed either a low dose of OVA (10×1 μ g), comparable with the amount of OVA secreted by the LL-OVA, or a high dose (1 $\times$ 200 mg or 5×1 mg) did not show a significant reduction in swelling compared with the control group. These data strongly indicate that LL-OVA can suppress systemic T-cell responses in DO11.10 mice and that this effect is

Table 1. In Vivo Detection of Live *L. Lactis* Bacteria

CFU LL-OVA	Distal small intestine	Cecum	Colon
Entire intestine, including luminal content	$7.25 \pm 0.14 \times 10^9$	$2.49 \pm 0.15 \times 10^9$	$3.49 \pm 0.015 \times 10^9$
Entire intestine, excluding luminal content	$4.16 \pm 0.25 \times 10^6$	$7.03 \pm 0.17 \times 10^7$	$1.41 \pm 0.10 \times 10^6$

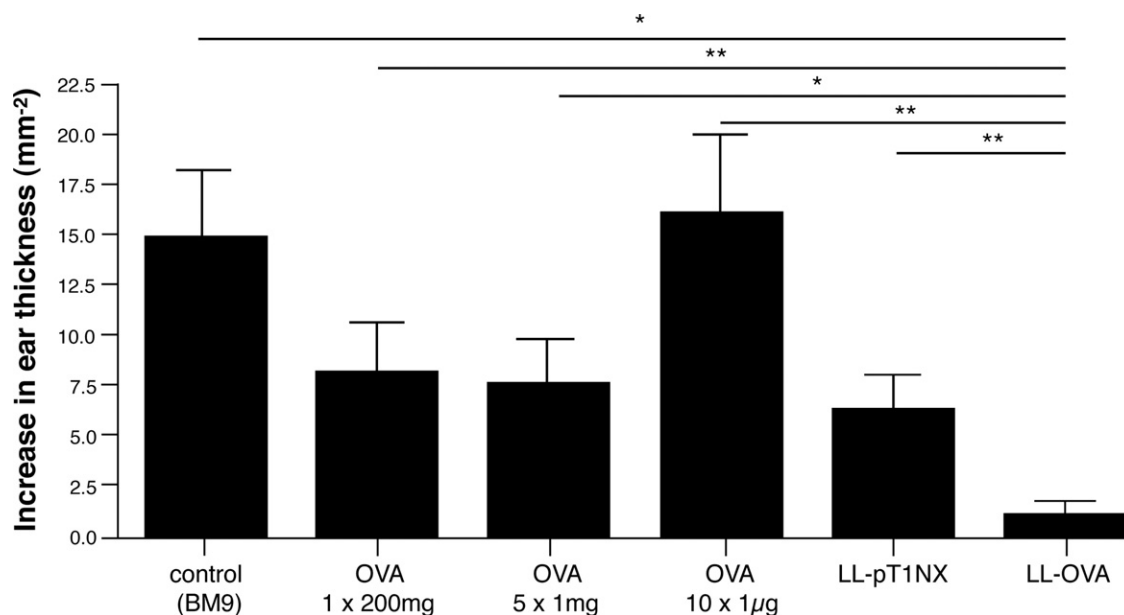


Figure 3. Oral feeding of LL-OVA significantly reduces DTH responses. DO11.10 mice were sensitized by subcutaneous injection of 100 µg OVA in CFA on day 1. Mice were orally treated with 200 mg OVA on day 8, 1 mg OVA on days 1–5, 1 µg OVA at days 1–5 and 8–12, or with LL-OVA or LL-pT1NX on days 1–5 and 8–12. Control mice received BM9. On day 11, mice were challenged with 10 µg OVA in 10 µL saline in the auricle of 1 ear and with 10 µL saline in the other. DTH responses are expressed as the mean difference in ear swelling between the OVA-injected and the saline-injected ears. Results summarize data of 4 independent experiments including 6 mice per group per experiment.

in part because of the apparent intrinsic tolerogenic effect of *L. lactis*.

Reduction of DTH Response by Treatment With LL-OVA Is Accompanied by an OVA-Specific Increase in IL-10 Production

Immediately after measuring DTH, cervical lymph node cells draining the ears of OVA-challenged mice were pooled and restimulated with OVA. This led to higher IL-10 production in the LL-OVA-treated mice than in the BM9 (control) and LL-pT1NX mice (Figure 4A).

IL-10 production by isolated bulk spleen and gut-associated lymph node cells (GALT) following 40-hour ex vivo OVA restimulation was also assessed immediately after DTH measurement. Production of IL-10 was significantly higher in restimulated bulk spleen cells from LL-OVA-treated mice than in the negative control and in the groups receiving 1 µg OVA or LL-pT1NX. Similar results were obtained using restimulated GALT cells (Figure 4B). The observed production of IL-10 was antigen specific because no differences in IL-10 production were observed after restimulation with anti-CD3/anti-CD28 (Figure 4C). In summary, we found that the production of IL-10 in OVA-stimulated cervical lymph nodes, splenocytes, and GALT cells was significantly higher in mice that were treated with LL-OVA.

Oral Feeding With *L. lactis* Suppresses the OVA-Specific Proliferative Capacity of Splenocytes and Decreases IFN-γ Production

Analysis of OVA-specific proliferation of splenocytes was used to examine peripheral immune responses.

Bulk splenocytes of mice treated with BM9, LL-pT1NX, or LL-OVA were isolated on day 12, and the OVA-specific proliferative response was assessed. Control mice were clearly sensitized and showed a high proliferative response. Daily intragastric administration of LL-OVA significantly reduced the OVA-specific proliferative response ($P = .002$) (Figure 5). Noteworthy, the OVA-induced proliferative response was also significantly reduced ($P = .022$) after intragastric administration of LL-pT1NX in comparison with the BM9 control group.

The lower proliferative response of spleen cells was accompanied by a significant down-regulation of IFN-γ production after ex vivo OVA restimulation, both in the LL-pT1NX- and the LL-OVA-treated mice (Figure 5, upper panel). These data indicate that LL-OVA and LL-pT1NX treatments are both able to suppress systemic T-cell responses in DO11.10 mice.

Feeding With LL-OVA Suppresses the OVA-Specific Proliferative Responses of CD4⁺ Splenic T Cells Mediated by TGF-β and CD4⁺CD25⁺ T Cells

To determine whether the reduced proliferative response of bulk splenocytes was related to reduced responsiveness of the CD4⁺ T cells, splenic CD4⁺ T cells from the mice treated with BM9, LL-pT1NX, or LL-OVA were isolated on day 12, and OVA-specific proliferative responses were assessed. The OVA-specific proliferative response of splenic CD4⁺ T cells was lower after LL-OVA treatment than in the BM9 and LL-pT1NX control groups (Figure 6A). Because blocking with neutralizing

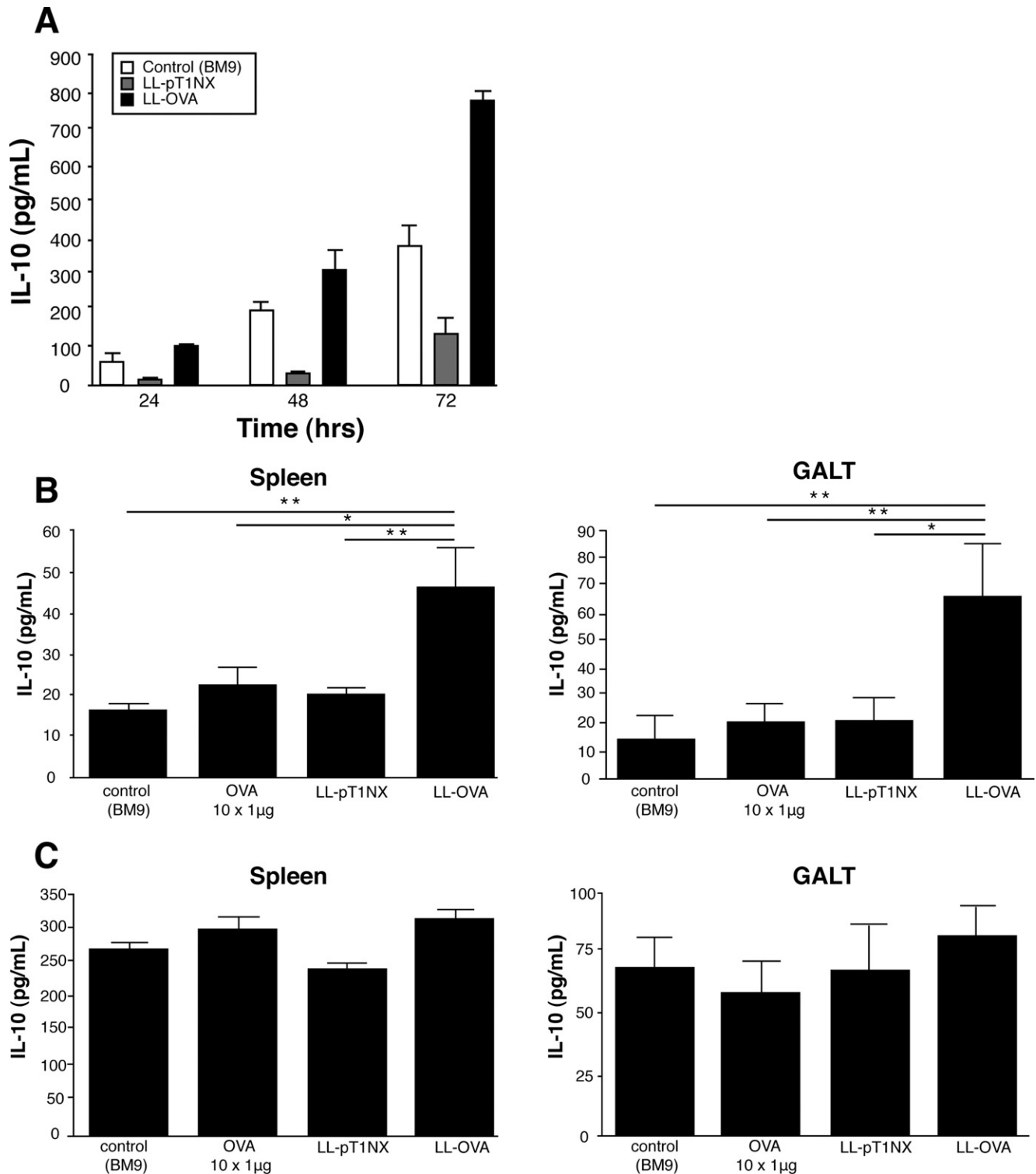


Figure 4. OVA-specific increase in IL-10 production after restimulation of cervical lymph node, spleen, and GALT cells. IL-10 secretion of pooled cervical lymph node cells was determined in the supernatants 24, 48, and 72 hours after restimulation. Data are represented as means of IL-10 secretion in pg/mL of at least 2 separate experiments (A). IL-10 production by bulk spleen and GALT cells was measured after ex vivo restimulation. Mice were fed BM9, 1 µg OVA, LL-pT1NX, or LL-OVA as described in Figure 3, and DTH was determined. Thereafter, bulk cell populations were isolated, and 1×10^5 cells were restimulated ex vivo with 0.5 mg/mL OVA (B). To determine antigen-specific IL-10 secretion, cells were restimulated with anti-CD3/anti-CD28 (C). Data summarize 3 independent experiments including 6 mice per experiment.

OVA-specific proliferation of bulk splenocytes

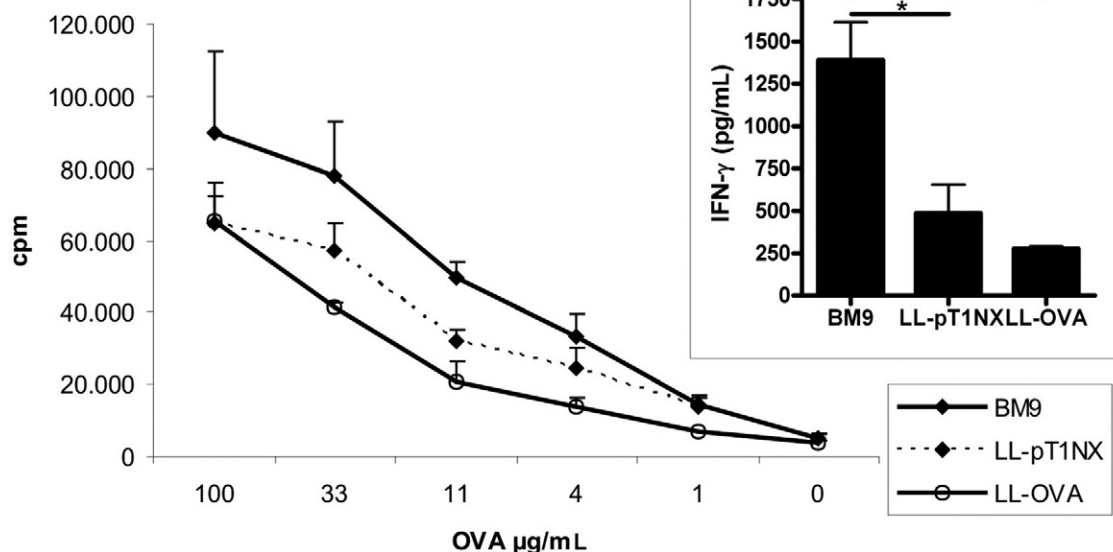


Figure 5. Feeding of LL-OVA or LL-pT1NX significantly reduces the OVA-specific proliferation and IFN- γ production of bulk splenocytes. DO11.10 mice were immunized by subcutaneous injection of 100 μ g OVA in CFA on day 1. Mice were treated orally with BM9 (control), LL-OVA, or LL-pT1NX on days 1–5 and 8–12. On day 12, bulk splenocytes were isolated and tested for OVA-specific proliferation, which is expressed as the mean cpm at different OVA concentrations, and for IFN- γ production after 72-hour ex vivo stimulation with 33 μ g/mL OVA (upper panel). Data represent at least 3 separate experiments, including 4 mice per group per experiment.

anti-IL-10 did not reverse the reduction in the proliferative response of bulk splenocytes (data not shown), we investigated whether TGF- β was involved. Blocking with TGF- β neutralizing monoclonal antibody did not significantly alter the proliferative response of the CD4⁺ splenic T cells in the BM9 and LL-pT1NX groups but

reversed the reduction in proliferation in the LL-OVA group.

Importantly, the reduction in proliferation because of LL-OVA treatment was maintained after depleting CD4⁺CD25⁺ T cells (Figure 6A). Moreover, the proliferative response of the CD4⁺CD25[−] T cells in the LL-OVA

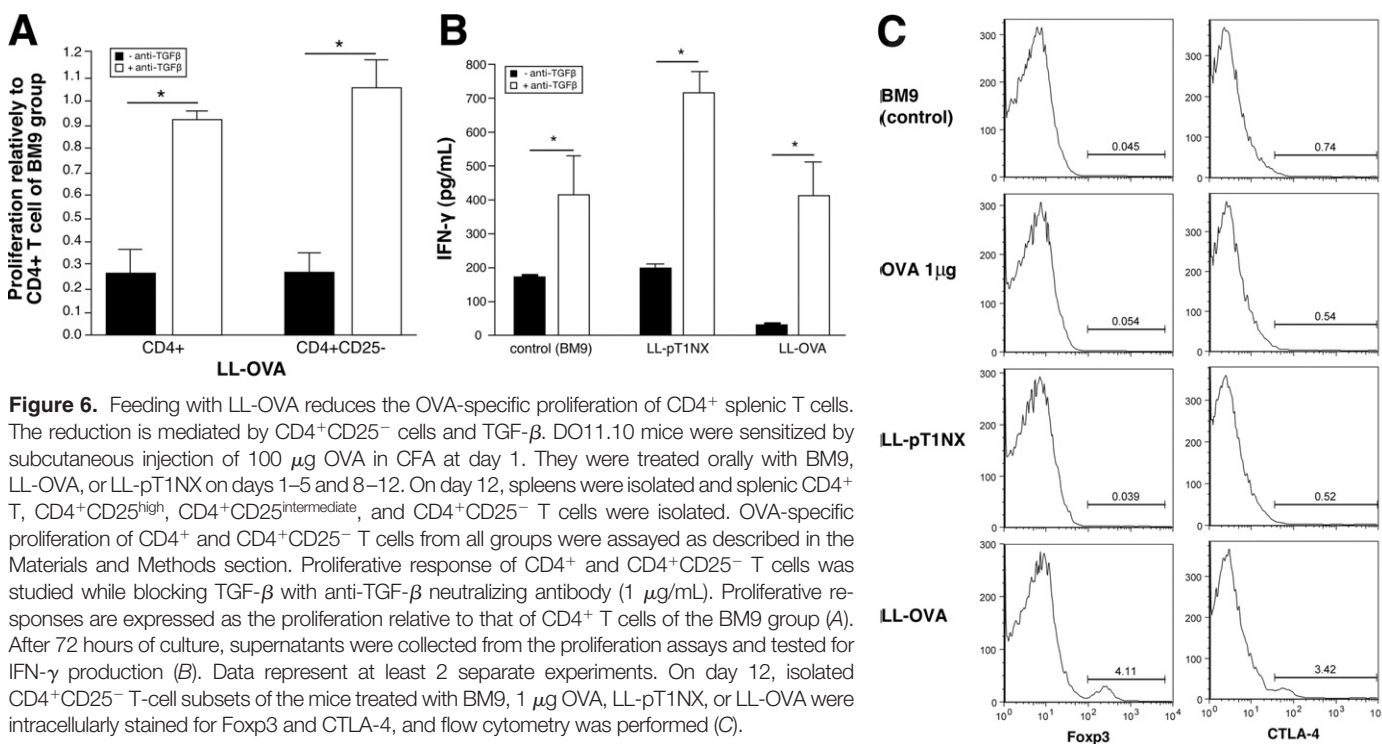


Figure 6. Feeding with LL-OVA reduces the OVA-specific proliferation of CD4⁺ splenic T cells. The reduction is mediated by CD4⁺CD25[−] cells and TGF- β . DO11.10 mice were sensitized by subcutaneous injection of 100 μ g OVA in CFA at day 1. They were treated orally with BM9, LL-OVA, or LL-pT1NX on days 1–5 and 8–12. On day 12, spleens were isolated and splenic CD4⁺ T, CD4⁺CD25^{high}, CD4⁺CD25^{intermediate}, and CD4⁺CD25[−] T cells were isolated. OVA-specific proliferative response of CD4⁺ and CD4⁺CD25[−] T cells from all groups were assayed as described in the Materials and Methods section. Proliferative response of CD4⁺ and CD4⁺CD25[−] T cells was studied while blocking TGF- β with anti-TGF- β neutralizing antibody (1 μ g/mL). Proliferative responses are expressed as the proliferation relative to that of CD4⁺ T cells of the BM9 group (A). After 72 hours of culture, supernatants were collected from the proliferation assays and tested for IFN- γ production (B). Data represent at least 2 separate experiments. On day 12, isolated CD4⁺CD25[−] T-cell subsets of the mice treated with BM9, 1 μ g OVA, LL-pT1NX, or LL-OVA were intracellularly stained for Fxp3 and CTLA-4, and flow cytometry was performed (C).

group significantly increased after blocking TGF- β with a specific neutralizing monoclonal antibody ($P = .028$). These proliferation data are supported by the IFN- γ production data (Figure 6B). To analyze further the induced CD4⁺CD25⁻ regulatory T cell (Treg) population, we defined the percentage of CD4⁺CD25⁻ lymphocytes expressing 2 major Treg markers: Foxp3 and CTLA4. For this purpose, we stained the isolated CD4⁺CD25⁻ T cells from mice treated with BM9 or 1 μ g OVA, LL-pT1NX, or LL-OVA on day 12 and gated them on the Foxp3 and CTLA4⁺ subpopulation. A de novo expansion of CD4⁺CD25⁻ Foxp3⁺ and CD4⁺CD25⁻ CTLA4⁺ is observed only in the LL-OVA-treated group (Figure 6C). This indicates that CD4⁺CD25⁻ regulatory T cells expressing Foxp3 and CTLA4 are induced following LL-OVA treatment and that, in vitro, the CD25⁻ cells mediate OVA-specific tolerance through a TGF- β -dependent mechanism.

CD4⁺CD25⁻ Regulatory T Cells Induced After LL-OVA Treatment Can Transfer OVA Tolerance in vivo

To assess the functional activity of the different CD4⁺CD25 subsets in vivo, we fed DO11.10 mice with LL-OVA as described above, and we adoptively transferred CD4⁺CD25^{high}, CD4⁺CD25^{intermediate}, and CD4⁺CD25⁻ subsets from their spleens into Balb/c mice sensitized by SC injection with 100 μ g OVA in CFA on days 0 and 1. Five days thereafter, mouse ears were injected with 10 μ g OVA, and ear thickness was measured 24 hours later. Remarkably, only adoptive transfer of the CD4⁺CD25⁻ T cells caused a reduction of DTH ($P = .0317$) (Figure 7). Together, these data strongly suggest that intragastric administration of OVA-secreting *L lactis* induces OVA-specific immune tolerance that is mediated by CD4⁺CD25⁻ regulatory T cells.

Discussion

Our data indicate that genetically modified *L lactis* can be used for mucosal delivery of antigens and that this suppresses local and systemic antigen-specific T-cell responses in both antigen-specific and nonspecific manners. Antigen-specific suppression induced by OVA-secreting *L lactis* is mediated by CD4⁺CD25⁻ "adaptive" Treg and seems to be dependent on TGF- β . Importantly, OVA dose feeding alone (either high dose or low dose) was less efficient than LL-OVA in reducing the DTH response in our model, indicating that the mode of mucosal delivery of an antigen to the immune system critically determines subsequent immune responses.

Induction of Treg is a major goal for immunotherapy for autoimmune diseases and several inflammatory diseases, and it can be achieved by exposing the mucosal immune system to low doses of antigen. It has been difficult to apply antigen-specific mucosal tolerance for the treatment of human diseases. Mucosal tolerance de-

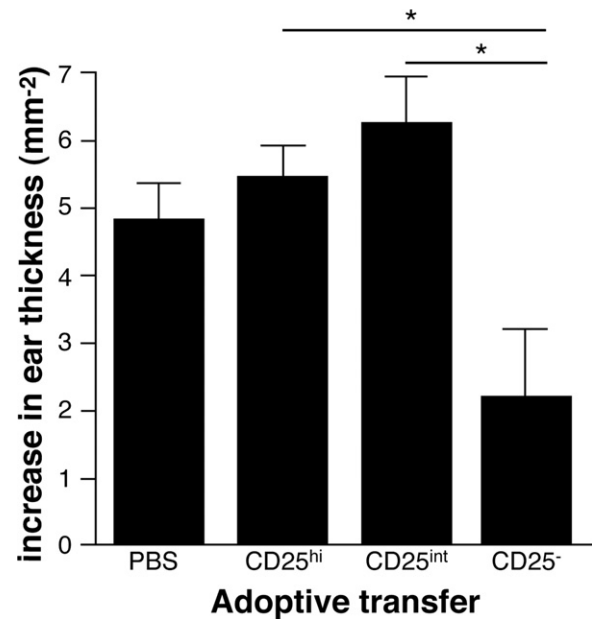


Figure 7. Adoptive transfer of CD4⁺CD25⁻ T cells of LL-OVA-treated mice into sensitized Balb/c mice induces OVA-specific tolerance in vivo. Three CD4⁺CD25 subpopulations were sorted: CD4⁺CD25^{high}, CD4⁺CD25^{intermediate}, and CD4⁺CD25⁻. On day 7, 5×10^4 cells of each subpopulation were adoptively transferred into Balb/c mice that had been sensitized by subcutaneous injection of 100 μ g OVA in CFA on days 0 and 1. Five days later, DTH measurements of the ear were performed.

pends critically on several factors, including the purity, source, and dose of antigen and the mode of antigen presentation to the mucosal immune system.⁴ Here, we propose a novel therapeutic strategy: active intestinal synthesis and delivery of an antigen by genetically engineered *L lactis*, which obviates the need for large scale purification of human (auto)antigens and circumvents current issues related to induction of oral tolerance in humans.

The in situ fate of the bacteria as well as the distribution of OVA produced by the LL-OVA was determined by multiple intragastric administrations to Balb/c mice. Except for the proximal part of the small intestine, viable *L lactis* were found throughout the intestine, with most bacteria located in the distal part of the small intestine and in the cecum. In a study performed by Droualt et al, only 10%–30% of the *L lactis* administered survived the duodenal transit.²³ Here, we show that more than 50% of the administered *L lactis* was recovered from the intestine. We believe that the BM9 inoculation buffer used in our experiments partially protects the bacteria against the extreme gastrointestinal conditions. The largest amounts of OVA were detected in the colonic lumen, with only small amounts in the intestinal tissue. Less OVA was found in the distal part of the small intestine, but much of it was in the intestinal tissue, indicating that the OVA is efficiently taken up by the small intestine. We have not studied uptake of OVA and/or *L lactis* by the distal small

intestine in detail, but it is possible that OVA peptide and/or lactococcal antigens were either taken up by M cells located in Peyer's patches or that whole *L lactis* were directly sampled by intraluminal extensions of mucosal DC.²⁴ Interestingly, most viable bacteria and the largest amounts of mucosal OVA were present in the distal small intestine, which is the predominant location of the intestinal sampling DC network.^{25,26} In addition, *L lactis* might serve as a bioadhesive delivery vehicle that delivers the antigen at the intestinal sampling network or intensifies contact with the mucosa. This would increase the antigen concentration gradient and ensure immediate absorption without dilution or degradation in the luminal fluid. The large difference between the immune responses to oral OVA and *L lactis*-delivered OVA indicates that these routes may be pivotal for induction of *L lactis*-mediated oral tolerance.

Both DC and Treg are critically involved in tolerance induction.²⁷⁻³¹ Our experiments demonstrate that *L lactis* influences APC-mediated, OVA-specific, T-cell proliferation because LL-OVA could induce OVA-specific proliferation, but OVA protein could not even at a concentration that was 2000-fold higher than that secreted by LL-OVA. Recently, we demonstrated that *L lactis* can imprint a regulatory phenotype on human monocyte-derived DC and that this effect is greatly boosted by engineered *L lactis* secreting IL-10 (Braat H, Steidler L, Neiryck S, Huibregtse I, Smits H, Zaat B, Kapsenberg ML, van Deventer S, de Jong E, manuscript submitted). Together, these data suggest that exposure to *L lactis* alters DC function, which in the presence of simultaneous exposure to a DC-presented antigen might result in the generation of an antigen-specific Treg subset.

We further demonstrated that both LL-pT1NX and LL-OVA reduce the DTH response and OVA-specific proliferation of bulk splenocytes and splenic CD4⁺ T cells. This reduction was not observed after feeding OVA protein, and it was much more pronounced in the LL-OVA than in the LL-pT1NX-treated mice. Moreover, it was only in the LL-OVA-treated mice that we observed antigen-specific production of IL-10 by OVA-stimulated cervical lymph node cells, splenocytes, and GALT cells, as well as TGF- β -dependent, OVA-specific, CD4⁺ T-cell suppression. Moreover, LL-OVA treatment induced within the CD4⁺CD25⁻ population a T-cell subset that expresses the typical Treg markers Foxp3 and CTLA-4. We also proved that the suppressive activity resident in the CD4⁺CD25⁻ Treg population could be transferred to sensitized Balb/c mice. These data indicate that *L lactis* can condition the mucosal immune system toward tolerance induction and boost antigen-specific induction of Treg by the codelivered antigen.

Different studies have demonstrated the efficacy of genetically modified *L lactis* to abrogate Th2-type responses induced in allergic mice models using nontransgenic Balb/c mice. However, in these studies the allergic

responses were diminished by the induction of counter-regulatory Th1 immune responses.³²⁻³⁵ In fact oral pretreatment of mice with natural *L lactis* plus soluble antigen or antigen-secreting *L lactis* abrogated the oral tolerance induced by antigen alone, demonstrating a Th1 adjuvant effect of these noncolonizing bacteria.³⁴ Apparently, *L lactis* has not such a Th1 adjuvant effect in DO11.10 OVA TCR transgenic mice. Recently, we demonstrated that the Th1 adjuvant effect of *L lactis* observed in nontransgenic Balb/c mice is absent in Balb/c mice fed over a long period with natural *L lactis* (Snoeck et al, manuscript in preparation). *L lactis* is not a normal constituent of the microflora or diet of laboratory mice. As such, it can be considered as a foreign microorganism in mice. In contrast, *L lactis* has been extensively consumed by humans and has never been associated with any form of pathology. Its main use lies in the manufacture of fermented milk, vegetable, and meat production. It is therefore granted a "generally regarded as safe" status, and, thus, it is acceptable to believe that *L lactis* will not have any Th1 adjuvant effect in humans. Currently, we are evaluating the *L lactis* delivery vehicle for the induction of antigen-specific immune tolerance in *L lactis* conditioned nontransgenic Balb/c mice.

Several phenotypically and functionally distinct Treg subsets have been implicated in suppression of intestinal inflammation and induction of oral tolerance.^{2,36,37} TGF- β has long been known to have a pivotal role in the induction of oral tolerance, both as a secreted cytokine and in the form of a latency-associated peptide corresponding to the aminoterminal domain of the TGF- β precursor protein.³⁸⁻⁴² Moreover, TGF- β -dependent Treg are efficiently induced by low doses of antigen.⁵ Previously, it has been demonstrated that oral tolerance induced by feeding OVA was only partly blocked by CD25 depletion but completely abrogated by blocking of TGF- β .⁴³ Recently, it was shown that antigen-specific, TGF- β -producing Th3 cells drive the differentiation of antigen-specific Foxp3⁺ regulatory cells in the periphery and play a crucial role in inducing and maintaining peripheral tolerance.⁴⁴ Although TGF- β partially mediates the suppressive effects of several types of Treg, both latency-associated peptide and (membrane-associated) TGF- β are intimately involved in the suppression mediated by a specific subset of GALT-derived CD4⁺CD25⁻ Tregs that can induce oral tolerance.⁴⁵ These cells suppress the colitis induced by transfer of CD4⁺CD45RB^{high} T cells into SCID mice,⁴⁶ and they are expanded after oral administration of low-dose anti-CD3 antibody to mice. Here, we demonstrate that oral administration of OVA-secreting *L lactis* induces a similar Treg population residing within the CD4⁺CD25⁻ compartment; but, in contrast to oral administration of anti-CD3 antibodies, the induced regulatory cells are antigen specific and Foxp3 and CTLA-4 positive.

The exact mechanism by which intestinal CD4⁺CD25⁻ Treg interfere with antigen-induced inflammatory reactions needs to be determined, and different possible mechanisms have been proposed. Effector T cells may be directly antagonized through the induction of immunosuppressive cytokines or by cell-cell contact, ie, by the activity of membrane-bound TGF- β .^{45,47} Suppression can also result via induction of "secondary" Treg that may depend on IL-10 production.^{48,49} Foxp3⁺CD25⁻ Th3 regulatory T cells represent a different cell lineage from thymus-derived CD25⁺ Tregs in the periphery but may play an important role in their maintenance.⁵⁰ Moreover, TGF- β -dependent conversion of peripheral CD4⁺CD25⁻ T cells into CD25⁺CD45RB^{-/low} suppressor cells has also been reported.⁵¹ The mechanism by which CD4⁺CD25⁻ cells function needs to be determined, but both orally administered anti-CD3 and LL-OVA-induced CD4⁺CD25⁻ regulatory cells clearly can suppress systemically induced inflammatory responses.

Therapeutic induction of Treg is a promising strategy for treating or restoring tolerance in patients suffering from autoimmune diseases. Current strategies for therapeutic induction of antigen-specific suppressor cells face considerable hurdles and usually require techniques to isolate, handle, and transfer adequate numbers of regulatory cells.^{9,10,52} The *L. lactis* antigen delivery system circumvents these problems and effectively induces antigen-specific Treg.

In conclusion, our data demonstrate that mucosal delivery of OVA by genetically modified *L. lactis* induces suppression of local and systemic OVA-specific T-cell responses in DO11.10 mice and that this effect does not depend solely on the secreted OVA but also on the presence of *L. lactis*. Oral administration of an antigen by *L. lactis* resulted in the induction of systemic tolerance mediated by CD4⁺CD25⁻ regulatory T cells that seem to function through a TGF- β -dependent mechanism. Importantly, the intestinal delivery system by *L. lactis* is superior to oral administration of soluble antigen because both low- and high-dose antigen feeding were not able to diminish the DTH response as significant as LL-OVA treatment in this model. These data indicate that engineered *L. lactis* could be an effective tool for inducing antigen-specific tolerance, with possible application in the treatment of antigen-induced autoimmune diseases.

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