Antigen-specific oral tolerance for the treatment of inflammatory and allergic diseases
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Mouse-strain specific immunogenicity of *Lactococcus lactis*: implications for the use of *L. lactis* antigen delivery system for oral tolerance induction

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Abstract

Background & Aims: The major goal of immunotherapy for autoimmune and allergic diseases is to obtain antigen-specific immune suppression mediated by regulatory T cells. Oral delivery of autoantigens or allergens by genetically modified Lactococcus lactis (L. lactis) would provide a novel therapeutic tool for such purposes. Recently, we have demonstrated that oral administration of OVA-secreting L. lactis can suppress systemic Th1 driven T cell responses by inducing regulatory T cells in OVA-immunized Balb/c mice and OVA TCR transgenic DO11.10 mice. On the other hand, different studies report a Th1 adjuvant property of L. lactis abrogating oral tolerance induced by the antigen alone. In the present study, we aimed to clarify this controversy, if any, and investigated the effect of L. lactis on Th1 driven inflammation when administered in a prophylactic setting. We further explored mouse strain differences in the immune response to L. lactis.

Methods: Balb/c and BL/6 mice received repeated small doses of OVA, L. lactis plus OVA or recombinant L. lactis secreting OVA (LL-OVA) by intragastric gavage during different feeding regimes. Subsequently, mice were challenged systemically by s.c. injection of OVA emulsified in complete Freund’s adjuvant to study the systemic immune response.

Results: In Balb/c mice, multiple oral L. lactis administrations in a prophylactic setting primed the Th1 responses against co-administered antigen. Oral feeding of L. lactis plus OVA and LL-OVA during short regimes primed the immune responses, i.e. increased OVA-specific proliferation as well as IFN-γ secretion upon in vitro OVA restimulation of lymphocytes, whereas administration of soluble OVA was tolerogenic. However, in longer feeding regimes this adjuvant effect declined. Simultaneously the L. lactis-specific IgG2a and IgA antibody responses in serum and feces increased, suggesting the generation of a tolerogenic state towards these bacteria. This was further supported by a tendency for reduced priming effect when the Balb/c mice were pretreated with L. lactis. In contrast to Balb/c mice, no Th1 adjuvant effect of L. lactis could be found in BL/6 mice. In comparison to LL-pTREX treated mice, oral feeding of LL-OVA significantly reduced the DTH response (P<0.1) and IFN-γ and IL-6 productions by the bulk PLN/ILN lymphocytes, whereas the production of IL-10 by splenic CD4+ T cells was significantly increased. This suggests a tendency for oral tolerance induction toward the orally delivered OVA.

Conclusion: Our data demonstrate differences in immunogenicity of L. lactis between Balb/c and BL/6 mice. Considering the initial Th1 adjuvant effect of the bacteria in Balb/c mice, these mice cannot be used for the evaluation of the L. lactis oral delivery technology for systemic
tolerance induction in prophylactic settings of Th1 pathologies. On the other hand, our data obtained in therapeutic settings using a Th1 driven OVA inflammation model, demonstrated that the Th1 adjuvant effect does not prevent the induction of regulatory T cells in antigen-sensitized conditions.

**Introduction**

A disturbed balance between tolerance toward harmless and self-antigens on the one hand and active immunity toward pathogenic agents on the other is a major cause of a wide variety of autoimmune and allergic diseases. The major goal of immunotherapy for these diseases is induction of regulatory T cells that mediate immunological tolerance. The mucosal immune system is unique as it has the ability to actively inhibit systemic immune responses to fed antigens, known as oral tolerance that is preferentially induced after exposure to harmless antigen. The induction of such an antigen-specific oral tolerance is an attractive therapeutic approach, because it generally lacks toxicity, can be easily administered over time, and avoids side effects associated with generalized immune suppressive intervention. However, whereas oral administration of (auto)antigens or allergens has been found effective in preventing the induction of autoimmune and allergic diseases in animal models, previous clinical attempts to induce oral tolerance for therapeutic purposes have been unsuccessful [1-3]. These failures are related to the source, the purity, and the amount of (auto)antigen needed and the presentation of the antigen to the mucosal immune system. Experimental data indicate that heterogeneous antigen mixtures are less effective in inducing oral tolerance than single purified antigens, and that the antigen dose is critical. Whereas high-dose antigen administration can lead to clonal deletion or anergy of T cells recognizing the antigen, low-dose antigen administration can result in induction of active suppression (e.g. antigen-specific regulatory T cells secreting suppressive cytokines) [1,4-6].

The *Lactococcus lactis* (*L. lactis*)-mediated delivery system offers an alternative for large-scale purification of human autoantigens or allergens, and enables delivery of antigens to the intestinal mucosa. *L. lactis* is a non-pathogenic, non-invasive, noncolonizing gram-positive bacterium. It is Generally Regarded As Safe (GRAS) according to the US Food and Drug Administration and is extensively used as fermenting agent in the food industry [7]. Genetically modified *L. lactis* strains for local synthesis and delivery of immunomodulatory proteins at the intestinal mucosa have been produced [8-9] and an adequate biological containment system for
its application in man has been established [10]. 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 suggested clinical efficacy [11]. Genetically modified L. lactis for intestinal delivery of proteins (human self-antigens or allergens) to induce oral tolerance is therefore a feasible strategy in man. Previously, it has been reported that L. lactis genetically modified to secrete allergen can be used to abrogate Th2-type responses induced in allergic models using Balb/c mice in both prophylactic and therapeutic settings [12-15]. However, in these models, the allergic responses were moderated by the induction of counter-regulatory immune responses, i.e. induction of a Th1 response, characterized by intense increase of antigen-specific IgG2a and IFN-γ secretion, and not by oral tolerance induction. In fact, in these mice oral pre-treatment with natural L. lactis plus soluble antigen or antigen-secreting L. lactis abrogates the oral tolerance induced by the antigen alone [13]. Furthermore, in the presence of lactic acid bacteria, monocyte-derived dendritic cells from allergic patients tend to reorientate the memory T cell response toward a beneficial Th1 profile, accompanied with a reduction in Th2 cytokine production [16]. Surprisingly, we could not demonstrate a Th1 adjuvant effect of these bacteria when they were administered in therapeutic settings in Th1-driven inflammatory models. On the contrary, we demonstrated that intragastric administration of OVA-secreting L. lactis suppressed local and systemic Th1-driven inflammatory T cell responses in OVA-immunized OVA-TCR transgenic DO11.10 mice, acting by inducing CD4⁺CD25⁻ regulatory T cells that function through a TGF-β dependent mechanism [17]. In these transgenic mice, L. lactis enhanced the tolerogenic signals, as the systemic inflammatory response was partially inhibited in mice receiving L. lactis alone [17]. Such a tolerogenic potential is in accordance with a study demonstrating that the bacteria can reduce the ability of bone marrow-derived DCs to activate allogeneic T cells proliferation [18]. Also in OVA immunized wild type Balb/c, administration of OVA-secreting L. lactis could significantly suppress the systemic Th1-driven T cell response by inducing LAP⁺CD4⁺CD25⁻ regulatory T cells [19]. However, in this model no immune regulatory functions of L. lactis were observed. Considering the controversy about the Th1 adjuvant characteristics of L. lactis, we aimed in the present study to further explore the feasibility of L. lactis as oral delivery vehicle for systemic tolerance induction in Th1 driven pathologies by investigating its effect on Th1 driven inflammation when applied in prophylactic settings in both Balb/c and BL/6 mice.
Material and methods

Bacterial strains, plasmids and growth conditions

The *Lactococcus lactis* MG1363 (LL) strain was genetically modified to secrete chicken ovalbumin as previously described [17]. *L. lactis*-pTREX 1, which is MG1363 carrying a plasmid encoding only for erythromycin resistance, served as empty vector control (LL-pTREX) [20]. Bacteria were cultured in GM17E medium i.e., M17 broth (Difco Laboratories, Detroit, MI) supplemented with 0.5% glucose and 5 µg/ml erythromycin (Sigma-Aldrich, Bornem, Belgium). Stock suspensions of LL strains were stored at -20°C in 50% glycerol in GM17E medium. Stock suspensions were diluted 500-fold in GM17E medium and incubated at 30°C overnight. Within 16 h they reached a saturation density of 2x10⁹ colony forming units (CFU) per ml. Bacteria were harvested by centrifugation and 10-fold concentrated in BM9 medium [8] at 2 x 10⁹ bacteria/100 µl. For treatment, mice received daily 100 µl of this suspension by intragastric catheter unless stated otherwise.

Mice oral pre-treatment and systemic immunization

Female Balb/c or BL/6 mice were obtained from Charles River Laboratories (Calco, Italy) and were housed in a conventional animal facility under specific pathogen-free conditions. Experiments were started at the age of 5 to 6 weeks. The animal studies were approved by the Ethics Committee of the Department for Molecular Biomedical Research at Ghent University (file no. 06/002 and 07/029).

Mice were fed 1 µg purified OVA (grade V, Sigma Aldrich) dissolved in 100 µl BM9, LL-pTREX+1 µg OVA, LL-OVA or LL-pTREX during different feeding regimes. Feeding regime 1 consisted of 2 cycles of daily administration for 5 days, alternating with a 2-days period of non-administrating. Feeding regime 2, 3 and 4 consisted of 4, 6 and 8.5 feeding cycles, respectively. Control mice were either non-fed (negative control group for OT induction) or received 20 mg purified OVA dissolved in 100 µl BM9 at day –7 (positive control group for OT induction). Single and multiple feedings were performed by intragastric administrations of antigen/bacteria using an 18-gauge stainless animal feeding needle. One day after the last feeding (day 0), mice were immunized subcutaneously at the base of the tail with 100 µg OVA emulsified 1:1 in complete Freund’s adjuvant containing 100 µg *Mycobacterium tuberculosis* H37 RA (Difco, Detroit, MI).
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Analysis of delayed-type hypersensitivity (DTH) response

Eleven days after immunization the DTH responses were assessed. Therefore, mice were challenged with 10 µg OVA in 10 µl saline in the auricles of the ears. Ear swelling, defined as the increase in ear thickness because of challenge, was measured 24 hours after challenge using a digital micrometer (Conrad, Oldenzaal, The Netherlands). DTH responses were expressed as the difference in ear-thickness before and after challenge for both ears.

Assessment of proliferative response and cytokine production

For proliferation assays and cytokine measurements, mice were sacrificed 12 days post-immunization or 8 days after ear challenge. Draining popliteal (PLN) and inguinal lymph nodes (ILN) on the one hand and spleens on the other hand were collected and pooled within each group. Single cell suspensions of lymph nodes and spleens were prepared by passing the cells through 70 µm filter cell strainers (Becton/Dickinson Labware). Erythrocytes in the spleen cell suspensions were lysed by incubation with red cell lysis buffer. CD4+ T cells were enriched using CD4 microbeads and midiMACS columns (all materials from Miltenyi Biotech, Germany). For proliferation assays of total lymphocyte populations, 2 x 10^5 cells were cultured in 96-well U-bottom plates (Becton Dickinson, Alphen aan de Rijn, The Netherlands) in a total volume of 200 µl complete medium [i.e. RPMI-1640 with 10% FCS, 2mM L-glutamax (Invitrogen, Merelbeke, Belgium), 0,4 mM sodium pyruvate, 50 µM 2-ME, and 10U/ml penicillin (Invitrogen) and 10 µg/ml streptomycin (Invitrogen)] either alone or with OVA. OVA was added at concentrations ranging from 1,2 to 100 µg/ml. To assess proliferation of CD4+ T cells, 2x10^5 CD4+ T cells were cultured with mitomycin C-treated OVA loaded splenocytes, acting as antigen-presenting cells at ratios of 1/0.3, 1/0.11, 1/0.03, 1/0.01, respectively. The cultures were grown in 96-well U-bottom plates (Costar, Sigma-Aldrich) in a total volume of 200 µl complete medium. Cells were cultured for 90 h at 37°C in a 5% CO2 humidified incubator. Proliferation was assessed by addition of 1 µCi/well [³H]-thymidin for the last 18 h of culture. DNA-bound radioactivity was harvested onto glass fiber filter mats (Perkin Elmer, Boston, USA) and thymidine-incorporation was measured on a scintillation counter (Perkin Elmer).

For cytokine assays, total lymphocyte and CD4+ T cell populations were cultured in the same way as in the proliferation assays. Culture supernatants were collected after 72 h and frozen at –70°C until cytokine analysis (IFN-γ, IL-6, IL-10, IL-4) was performed. Cytokine production
was quantified using the Mouse Inflammation Cytometric Bead Assay (BD Biosciences, Erembodegem, Belgium).

**Serum antibody detection**

For antibody assays, mice were bled under anaesthesia from the auxiliary plexus 12 or 21 days post-immunization or 8 days after ear challenge and serum was collected for antibody detection. Anti-OVA antibody titers were determined by standard ELISA. Microtiter plates (Maxisorp, Nunc, VWR International, Haasrode, Belgium) were coated with OVA by adding 50 µl of protein solution [2 µg/ml in phosphate-buffered saline (PBS, pH 7.6)] to each well and incubating the plates overnight at 4°C. Subsequently, the wells were blocked with 200 µl 0.1% casein in PBS for 2 h at 37°C. After washing with PBS containing 0.05% Tween 20 (PBS-T), the plates were incubated for 1 h at 37°C with 50 µl of mouse serum samples diluted 1:10 to 1:20480 in PBS containing 0.1% casein and 0.05% Tween 20 (PBS-CT). After washing with PBS-T, the plates were incubated with 50 µl goat anti-mouse IgG2a-HRP [Southern Biotechnology Associates (SBA), Imtec ITK Diagnostics, Antwerpen, Belgium, dilution 1:5000] or goat anti-mouse IgG1-HRP (SBA, dilution 1:5000) for 1 h at 37°C. After washing, 50 µl substrate [3,3',5,5' tetramethylbenzidine (TMB) substrate reagent, Pharmingen, Becton Dickinson, Erembodegem, Belgium] was added to each well. Finally, reactions were stopped by adding 1M H2SO4 to the wells. The absorbances were read at 450 nm. ELISA scores are expressed as titers, which are the inverse of the highest dilution that still had an OD450 higher than the calculated cut-off value. The cut-off was calculated as the mean OD450 of 5 non-immunized mice increased with three times the SD.

Anti-*L. lactis* specific antibody titers were determined by standard ELISA, using *L. lactis* sonicate. Hereto, LL-pTREX stock suspension was diluted 500-fold in GM17E medium and incubated at 30°C overnight. Bacteria were harvested by centrifugation and 20-fold concentrated in PBS. After addition of DNase (10 µg/ml), this suspension was sonicated 3 times for 5 min on ice, using a solid probe (3/4”) and a high intensity ultrasonic processor (Analys, Suarlée, Belgium). The suspension was then centrifuged at 10,000 x g for 10 min. The supernatant was collected, sterile filtered and stored at −20°C until use. The protein concentration in the supernatant was typically 2.0-2.5 mg/ml, as determined by advanced protein assay reagent (Cytoskeleton, tebu-bio, Belgium). Microtiter plates were coated with 50 µl 2 µg/ml LL-pTREX sonicate overnight at 4°C. Subsequently, the wells were blocked, incubated with mouse serum samples, goat anti-mouse IgG2a-HRP/IgG1-HRP or IgA-HRP antibodies,
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TMB substrate reagent and 1M H$_2$SO$_4$ as described above for the OVA-specific ELISA. The ELISA scores are expressed as titers. The cutoff was calculated as the mean OD$_{450}$ of the mice of the water group, the 1 µg OVA and 20 mg OVA group increased with three times the SD.

Statistical analysis

OVA-specific proliferation was tested for significance using a general linear model with repeated measurements. Statistical significant differences between the different groups in serum titers and cytokine production were assessed using the one-way Anova test. Statistical significance is indicated as * P<0.05 and **P<0.01.

Results

**L. lactis primes the systemic Th1 response against co-administered antigen in short feeding regimes in Balb/c mice**

Analysis of OVA-specific proliferation upon *in vitro* stimulation of draining inguinal (ILN) and popliteal (PLN) lymph node cells after an oral feeding regime is used to examine oral tolerance induction. We have recently modified *L. lactis* to secrete chicken ovalbumin [17]. OVA was secreted *in vitro* in the ng range and was actively delivered at the intestinal mucosa by oral administration of this *L. lactis* strain. Mice received 1 µg OVA, LL-pTREX+1 µg OVA, LL-OVA or LL-pTREX during different feeding regimes. Feeding regime 1 and 2 consisted of 2 and 4 feeding cycles, respectively. Non fed mice were used as control. Mice that were fed once with 20 mg OVA served as positive control group for oral tolerance induction. After feeding, mice were systemically challenged by s.c. injection of OVA in CFA. Twelve days post-immunization, ILN and PLN cells were isolated and the OVA-specific proliferative response was assessed.

Control mice were significantly sensitized and their T cells showed a high proliferative response upon exposure to OVA (fig. 1). Mice that were fed once with 20 mg OVA exhibited oral tolerance, since they showed a significantly reduced OVA-specific proliferative response. In both feeding regime 1 and 2, no differences in the OVA-specific proliferative responses were found between the control mice and LL-pTREX treated mice, whereas the proliferative response was significantly enhanced after intra-gastric administration of both LL-OVA and LL-pTREX+1 µg OVA (Fig. 1). Whereas in feeding regime 1 no difference was found between the control mice and the 1 µg OVA treated mice (Fig. 1a), oral intra-gastric administration of 1 µg OVA during feeding regime 2 significantly reduced the OVA-specific proliferative response (Fig. 1b). These data demonstrate that intra-gastric
Figure 1. In Balb/c mice, intra-gastric administration of LL-OVA or LL-pTREX+1µg OVA during feeding regimes 1 and 2 significantly enhances the OVA-specific proliferative response of ILN and PLN cells. Balb/c mice were fed 1 µg OVA, LL-pTREX+1 µg OVA, LL-OVA or LL-pTREX during feeding regime 1 (Fig 1a) or 2 (Fig. 1b). Non-fed mice were used as control. Mice fed once 20 mg OVA served as positive control group for tolerance induction. In a separate experiment mice were fed LL-pTREX+ 1 µg OVA at a daily dose of 2x10^9 bacteria or 2x10^7 bacteria during feeding regime 2 (Fig.1b insert). After feeding the mice were immunized with 100 µg OVA in CFA. Twelve days post-immunization, draining inguinal and popliteal lymph nodes were isolated and tested for OVA-specific proliferative response that is expressed as the mean cpm ± SEM in function of different OVA concentrations. Data represent 2 separate experiments, including 4 animals per experiment.
Figure 2. IFN-γ production after *in vitro* restimulation with OVA in ILN and PLN cell cultures from Balb/c mice treated with OVA or *L. lactis* during regime 1 and 2. Balb/c mice were fed 1 µg OVA, LL-pTREX+ 1 µg OVA, LL-OVA or LL-pTREX during feeding regime 1 (Fig. 2a) or 2 (Fig. 2b). Non-fed mice were used as control. Mice fed once 20 mg OVA served as positive control group for tolerance induction. After feeding the mice were immunized with 100 µg OVA in CFA. Twelve days post-immunization, draining ILN and PLN were isolated. IFN-γ secretion of ILN and PLN cells was determined in the supernatants 72 h after restimulation with 33 µg/ml OVA (feeding regime 1) or 100 µg/ml OVA (feeding regime 2). Data represent 2 separate experiments, including 4 animals per experiment.
Th1 adjuvant effect

administration of *L. lactis* bacteria during these short feeding regimes primes the systemic Th1 response against the co-administered OVA, as both LL-OVA and LL-pTREX+1 µg OVA enhances the systemic OVA-specific T-cell response and abrogates the tolerance induction towards OVA. Lowering the daily dose of LL-pTREX from 2x10⁹ to 2x10⁷ bacteria during feeding regime 2 did not abrogate the priming effect of *L. lactis* (Fig 1b insert). Also the use of heat killed or UV killed bacteria instead of viable LL-pTREX did not diminish the adjuvant effect of *L. lactis* (data not shown). Taken together, these data demonstrate that the adjuvant effect of *L. lactis* is independent on viability of the bacteria and is not diminished by lowering the dose 100 times.

These proliferative data are supported by IFN-γ measurements (Fig. 2). ILN and PLN cell cultures of mice pre-treated during regime 2 with LL-OVA and LL-pTREX+1 µg OVA released significantly more IFN-γ after reactivation with OVA than cell cultures from control mice. However, no significant increase in IFN-γ production was observed after pre-treatment during regime 1. Lower IFN-γ production was measured in the mice treated with 20 mg OVA. IL-4 production was minimal in all groups (< 40 pg/ml) (data not shown), confirming the Th1 polarization of the immune response induced in this model by s.c. immunization with OVA in CFA.

To examine the effect of the different treatments on humoral immunity, the OVA-specific serum antibody response was tested. Strong IgG1 and IgG2a antibody responses were observed in all groups after feeding during regime 1 and 2 (Fig. 3). Although significance was not reached, the OVA-specific IgG1 and IgG2a responses were slightly increased in the LL-OVA and LL-pTREX+1 µg OVA treated mice in feeding regime 2. After feeding during regime 1, a small increase of the IgG2a response was observed in the LL-OVA treated mice. Slightly lower OVA-specific titers were measured in mice fed 20 mg OVA. The results show that none of the treatment protocols resulted in tolerization for OVA-specific antibody responses and oral administration of *L. lactis* treatment slightly boosted the antibody response, as the antibody titers were slightly increased after treatment with LL-OVA and LL-pTREX+1 µg OVA during regime 2. However, the IgG1/IgG2a titer ratio did not demonstrate a specific polarization towards a Th1 response in the LL-OVA and LL-pTREX+1 µg OVA treated mice.
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The adjuvant effect of *L. lactis* in Balb/c mice declines in longer feeding regimes

We next analysed the OVA-specific proliferation upon *in vitro* restimulation of draining inguinal (ILN) and popliteal (PLN) lymph node cells in longer feeding regimes. In regime 3, consisting of 6 feeding cycles, the OVA-specific proliferative response was significantly reduced in mice fed once 20 mg OVA or multiple doses of 1 µg OVA, indicative for an induced tolerogenic state. In contrast, feeding of LL-pTREX, LL-pTREX+1µg OVA or LL-OVA did not enhance or decrease the OVA-specific proliferative response (Fig. 4). The difference in proliferative responses were paralleled by changes in IFN-γ production by PLN and ILN cells of mice treated with 20 mg, 1 µg OVA, LLpTREX and LL-pTREX+1µg OVA. However, despite the absence of an enhanced proliferative response in the LL-OVA treated mice, the IFN-γ production by the PLN and ILN cells was still significantly higher in

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Figure 3. Slightly increased OVA-specific antibody levels in sera of Balb/c mice treated with LL-OVA and LL-pTREX+1 µg OVA after treatment during regime 2. Balb/c mice were fed 1 µg OVA, LL-pTREX+ 1 µg OVA, LL-OVA or LL-pTREX during feeding regime 1 or 2. After feeding the mice were immunized with 100 µg OVA in CFA. Twenty-one days post-immunization, serum was collected and tested for OVA-specific IgG1 and IgG2a antibodies. Antibody levels are reported as mean titers ± SEM. Data represent 2 separate experiments, including 4 animals per experiment.
Th1 adjuvant effect

Comparison to the control group (Fig. 5). These data demonstrate that the adjuvant effect of *L. lactis* observed in the short feeding regimes declines in feeding regime 3, as no enhanced proliferative response is observed in LL-OVA and LL-pTREX+1µg OVA treated mice.

Oral *L. lactis* treatment induces *L. lactis*-specific serum IgG2a and IgA antibodies in Balb/c mice

We further explored whether *L. lactis* treatment induces a *L. lactis*-specific antibody response. A low *L. lactis*-specific IgG2a serum antibody response was induced after feeding during regime 1- and this only in the LL-pTREX treated mice (data not shown), that increased in the longer feeding regimes (Fig. 6). No *L. lactis* specific IgG1 antibodies could be detected, demonstrating that a humoral Th1 response is induced by lactococcus components. Furthermore, also low *L. lactis*-specific IgA antibodies could be detected from feeding regime 2 onwards in serum and from feeding regime 4 in the feces (Fig. 6).
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Figure 5. IFN-γ production after in vitro restimulation with OVA in ILN and PLN cell cultures from Balb/c mice treated with OVA or L. lactis during regime 3.
Balb/c mice were fed 1 µg OVA, LL-pTREX+ 1 µg OVA, LL-OVA or LL-pTREX during feeding regime 3. Control mice were not treated. Mice fed once 20 mg OVA served as positive control group for tolerance induction. After feeding the mice were immunized with 100 µg OVA in CFA. Twelve days post-immunization, draining ILN and PLN were isolated. IFN-γ secretion of ILN and PLN cells was determined in the supernatants 72 h after restimulation with 33 µg/ml OVA. Data represent 2 separate experiments, including 4 animals per experiment.

The adjuvant effect of L. lactis found in Balb/c mice is lost when mice are orally pre-treated with L. lactis
As the adjuvant effect of L. lactis disappeared in longer feeding regimes, we investigated whether this could be attributed to an induced tolerogenic state towards L. lactis in these regimes. Therefore, we treated the mice intra-gastrically with LL-pTREX using regime 3, after which a rest period of 60 days was introduced. Subsequently the mice were fed using regime 2 with LL-OVA, after which they were immunized s.c. with OVA in CFA as described in material and methods. Twelve days post-immunization, ILN and PLN cells were isolated and the OVA-specific proliferative response was assessed and compared to the proliferative response of control mice and mice treated with LL-OVA during regime 2 without pre-treatment with LL-pTREX.
Control mice were significantly sensitized and showed a high proliferative response. This response was significantly enhanced after intra-gastric
Figure 6. *L. lactis* treatment induces an *L. lactis*-specific IgG2a and IgA antibody response in Balb/c mice.

Balb/c mice were fed 1 µg OVA, LL-pTREX+ 1 µg OVA, LL-OVA or LL-pTREX during feeding regime 2, 3 or 4. Control mice not treated or received once 20 mg OVA. After feeding the mice were immunized with 100 µg OVA in CFA. Serum was collected 21 (regime 2 and 3) or 12 (regime 4) days post-immunization and tested for *L. lactis*-specific IgG2a and IgA antibodies. Antibody levels are reported as mean titers ± SEM. Data represent 2 separate experiments, including 4 animals per experiment.

administration of LL-OVA during regime 2, confirming the adjuvant effect of *L. lactis* observed in short feeding regimes (Fig. 7a). However, when the mice were pre-treated with LL-pTREX, no enhancement of the OVA-specific proliferative response was observed after LL-OVA treatment during regime 2. These proliferative data are supported by IFN-γ production by ILN and PLN cell cultures (Fig. 7b). Analysis of the *L. lactis*-specific serum IgG2a response revealed significantly higher titers after pre-treatment with *L. lactis* (Fig. 7c). This demonstrates that the adjuvant effect of *L. lactis* is lost in *L. lactis* conditioned mice and suggests that the adjuvant effect observed in short feeding regimes can be attributed to a non-tolerogenic state of the mice towards *L. lactis* bacteria.

**LL-OVA feeding during a short feeding regime does not prime the OVA-specific immune response in BL/6 mice**

To investigate whether the adjuvant effect of *L. lactis* observed in short feeding regimes in Balb/c mice was mouse strain-specific, BL/6 mice
were fed during feeding regime 2 with LL-OVA or LL-pTREX. Control mice received water. After feeding, the mice were systemically challenged by s.c. injection of OVA in CFA. Eleven days post-immunization, the DTH response was analysed. One week after the DTH analysis, the PLN, ILN and spleens were isolated to assess the OVA-specific proliferative response and cytokine production. In these mice, the DTH response was weakly (P<0.1) reduced after LL-OVA in comparison to LL-pTREX treatment (Fig 8). Whereas no significant differences could be found in proliferative response of the bulk PLN and ILN lymphocytes, significantly lower IFN-γ and IL-6 was produced in the LL-OVA treated group (Fig. 9). Remarkably, both feeding of LL-OVA and LL-pTREX significantly reduced
Th1 adjuvant effect

The proliferative response and production of IL-10, IL-6 and IFN-γ of bulk splenocytes (Fig. 10). A focus on the splenic CD4+ T cells revealed in the *L. lactis* treated groups, a higher proliferative response of these cells that was accompanied with a significantly lower production of IL-6 and IFN-γ (Fig 10). Remarkably, only in the LL-OVA treated group the production of IL-10 was intensely increased (Fig 10). Analysis of the OVA-specific serum IgG1 and IgG2a titers revealed no significant differences between the different groups (data not shown).

Discussion

Our data indicate that mucosal administration of *L. lactis* during short feeding regimes in Balb/c mice primes the Th1 response against the co-administered antigen, abrogating oral tolerance induction. Repetitive oral administration of 1 µg OVA during feeding regime 2 resulted in a significant reduction of the OVA-specific proliferative response of ILN and PLN cells, indicative for tolerance induction, which was abrogated by addition of either *L. lactis* or OVA-secreting *L. lactis*. These findings are in agreement with previous studies demonstrating the efficacy of *L. lactis*...
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to induce counter-regulatory Th1 responses in models of allergy using Balb/c mice [13-15]. This was attributed to the strong intrinsic capacity of *L. lactis* to induce *in vitro* Th1-promoting cytokines IL-12 and IFN-γ from spleen cells, isolated from Balb/c mice [15]. We observed a slightly higher IFN-γ production after administration of LL-OVA in comparison to LL-pTREX+1 µg OVA treatment. This may be explained by a higher local OVA concentration at the intestinal immunological inductive sites after LL-OVA than after 1 µg OVA administration, the latter being probably more sensitive to digestion by gastric pepsin and intestinal luminal peptidases compared to OVA locally produced by *L. lactis*. This is in agreement with a previous study [13] that demonstrated that the magnitude of the Th1 polarisation is dependent on the production level of the antigen, the higher the antigen produced the higher the Th1 polarisation, i.e. IgG2a antibodies and IFN-γ production. In our study, oral pretreatment with LL-OVA and LL-pTREX+1 µg OVA was not accompanied by a significantly increased production of OVA-specific IgG2a antibodies. This can be attributed to the low OVA concentrations used (1 µg) or produced by LL-OVA (ng range). Indeed, previously it has been demonstrated that oral administration of low antigen (4 µg) producing *L. lactis* strains or *L. lactis* + 50 µg antigen cannot induce high levels of specific IgG2a antibodies and are unable to induce a humoral shift towards a Th1 response [13].

Figure 8. Oral administration of LL-OVA during feeding regime 2 to BL/6 mice slightly reduced the DTH response in comparison to LL-pTREX treated mice.
BL/6 mice were fed LL-OVA or LL-pTREX during feeding regime 2. Control mice were not treated. After feeding the mice were immunized with 100 µg OVA in CFA. Eleven days post-immunization, mice were challenged with 10 µg OVA in 10 µl saline in the auricles of the ears. DTH responses are expressed as the difference in ear swelling before and after OVA challenge.
Remarkably, in longer feeding regimes, the adjuvant effect of *L. lactis* found in Balb/c mice disappeared. Simultaneously, the production of *L. lactis*-specific IgG2a and IgA antibodies in serum and/or feces increased. This suggest that a tolerogenic state towards *L. lactis* is induced in Balb/c mice during longer feeding regimes. IgA antibodies towards commensals that contain these bacteria within the lumen of the intestine and prevent their systemic dissemination [21] are developed during tolerance induction, supporting this hypothesis. Furthermore, oral pretreatment of Balb/c mice with *L. lactis* interfered with the ability of *L. lactis* to prime
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Figure 10. In BL/6 mice, *L. lactis* treatment during feeding regime 2 significantly reduced the proliferative response and cytokine production of bulk splenocytes. Analysis of the CD4+ splenic T cells demonstrated an increased proliferative response and decreased production of IL-6 and IFN-γ in both *L. lactis* treated groups. Only LL-OVA treatment significantly increased the production of IL-10 by CD4+ splenic T cells.

BL/6 mice were fed LL-OVA or LL-pTREX during feeding regime 2. Non-fed mice were used as control. After feeding th mice were immunized with 100 µg OVA in CFA. Eleven days post-immunization the mice were challenged with 10 µg OVA in 10 µl saline in the auricles of the ears. Seven days after ear challenge, spleens were collected. The proliferative response and cytokine production of bulk splenocytes and CD4+ splenic T cells were analysed after 90 h and 72 h after *in vitro* restimulation, respectively.
the Th1 response, demonstrating that the Th1 adjuvant effect is lost in *L. lactis* desensitized mice.

Our data further demonstrate that in contrast to Balb/c mice, no Th1 adjuvant property of *L. lactis* could be demonstrated in BL/6 mice. This suggests either strain-specific immunogenicity of *L. lactis* components or either predominant induction of different T cell subsets (Th1 versus Th2) depending on the MHC class II genotype of the responding mice [22]. Heterogeneity in the immunogenic potential of antigens between mouse strains has previously been demonstrated [23]. Such an influence of the genetic make-up of different mouse strains on the immune response is explained by the existence of strain-dependent immunodominant regions on the antigen [23].

From our data, it becomes clear that *L. lactis* exert a Th1 adjuvant activity on the immune response to co-administered antigen when it is administered in a prophylactic setting during short feeding regimes in Balb/c mice. This agrees with different studies, reporting the usefulness of genetically modified *L. lactis* to abrogate Th2-type responses induced in allergic models using non-transgenic Balb/c mice by the induction of counter-regulatory immune responses [13-15]. The different feeding regimes examined in the present study enabled us to extend their findings and the results indicate that the Th1 priming effect of *L. lactis* declines in longer feeding regimes, which can probably be explained by the generation of a tolerogenic state toward *L. lactis*. One of the key issues in oral tolerance induction is to prevent activation of immune responses that may enhance adverse symptoms. Considering the initial Th1 priming, Balb/c mice can not be used for the evaluation of the *L. lactis* oral delivery technology for oral tolerance induction in prophylactic settings of Th1 pathologies. On the other hand, in a Th1-driven OVA inflammation model using Balb/c mice, we were able to demonstrate induction of OVA-specific immune tolerance by intragastric administration of OVA-secreting *L. lactis* in a therapeutic setting. The suppressive effect was mediated by CD4^+^CD25^−^LAP^+^ regulatory T cells [19]. Apparently, the Th1 adjuvant property of *L. lactis* in Balb/c mice does not cumber the induction of oral tolerance in therapeutic settings. Consequently, whereas not useful in a prophylactic setting, Balb/c mice can be used for the evaluation of oral tolerance induction in therapeutic settings of Th1-driven pathologies. Since no Th1 adjuvant potential of *L. lactis* was demonstrated in a prophylactic setting in BL/6 mice, they can be considered as the mouse strain of choice for evaluation of *L. lactis* as antigen delivery vehicle for oral tolerance induction purposes in prophylactic settings.

In conclusion, considering the difference in immune modulatory properties (Th1 versus tolerogenic) of *L. lactis* between different mouse
strains (Balb/c, BL/6, OVA TCR transgenic mice) and the decline in Th1 adjuvant effect in longer feeding regimes in Balb/c mice, data on Th1 counter-regulatory potential of *L. lactis* obtained in prophylactic settings using Balb/c mice that do not normally ingest this bacterium, need to be carefully extrapolated to humans. Whether *L. lactis* will have immune regulatory effects in humans has not been evaluated yet and can only be answered by clinical trials. Secondly, together with our study performed in therapeutic settings both in OVA TCR transgenic and wildtype Balb/c mice, these results hold promise that active delivery of antigens by genetically modified *L. lactis* can provide a novel tool for the induction of systemic tolerance in Th1-driven autoimmune diseases by inducing regulatory T cells.

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**References**


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