Antigen-specific oral tolerance for the treatment of inflammatory and allergic diseases

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Induction of antigen-specific tolerance by oral administration of *Lactococcus lactis* delivered immunodominant DQ8-specific Gliadin peptides in sensitized NOD AB\(^{0}\) DQ8 transgenic mice

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Abstract

Background and aims: Active delivery of recombinant autoantigens or allergens at the intestinal mucosa by genetically modified Lactococcus lactis (LL) provides a novel therapeutic approach for the induction of tolerance. Celiac disease is associated with either DQ2 or DQ8 restricted responses to specific antigenic epitopes of gliadin, and may be treated by induction of antigen-specific tolerance. We investigated whether oral delivery of a DQ8 epitope delivered by LL induces antigen-specific tolerance.

Methods: L. lactis was engineered to secrete a deamidated DQ8 epitope (LL-eDQ8d) and the induction of antigen-specific tolerance was studied in NOD AB\textsuperscript{o} DQ8 transgenic mice. Tolerance was assessed by delayed-type hypersensitivity reaction, cytokine measurements, eDQ8d-specific proliferation and regulatory T cell analysis.

Results: Oral administration of LL-eDQ8d induced suppression of local and systemic DQ8 restricted T-cell responses in NOD AB\textsuperscript{o} DQ8 transgenic mice. Treatment resulted in an antigen-specific decrease of the proliferative capacity of the splenocytes and inguinal lymph node cells, which was critically dependent on the production of IL-10 and TGF-\textbeta and associated with a significant induction of Foxp3\textsuperscript{+} regulatory T-cells.

Conclusions: These data provide support for the development of effective therapeutic approaches for celiac disease using orally administered antigen-secreting L. lactis. Such treatments may be effective even in the setting of established hypersensitivity.

Introduction

The chronic small intestinal inflammation that defines celiac disease is characterized by flattening of the villous architecture and massive infiltration of T cells which release proinflammatory cytokines, such as IFN-\gamma and IL-2. Celiac disease is caused by a loss of tolerance to ingested dietary gliadin and is mediated by a HLA-DQ2 or HLA-DQ8 restricted T-cell response\textsuperscript{1}. Effective treatment can only be reached by a socially restrictive diet that requires lifelong abstinence from foods that contain dietary gliadin present in wheat or proteins from related cereals like rye or barley. While a strict gluten free diet can lead to healing of the intestine, the intolerance to gluten is permanent\textsuperscript{2} and better therapeutic options are needed\textsuperscript{3}.

Oral tolerance is defined as the induction of antigen-specific suppression of immune responses to an antigen by its prior oral feeding and is an
eDQ8d tolerance

attractive therapeutic approach for treatment of allergic, autoimmune and inflammatory diseases. In contrast to most other autoimmune diseases, the trigger, the genetic association, and the highly specific humoral response have been well characterized for celiac disease. Because disease activity is strongly correlated to the presence and dosage of antigen, the induction of antigen-specific oral tolerance is an attractive therapeutic approach. Oral tolerance is mediated by multiple mechanisms such as anergy, deletion and/or active suppression of antigen-specific effector T cells by regulatory T cells (Treg). The efficacy of oral tolerance in preventing the induction of autoimmune and allergic diseases has been clearly demonstrated in several animal models, but unfortunately previous clinical attempts to induce oral tolerance for therapeutic purposes have failed. These failures are related to the source, the purity, and the amount of (auto)antigen needed and the mode of presentation of the antigen to the mucosal immune system. Previously, a delivery system (TopAct™) based on living Lactococcus lactis strains (ActoBiotics™) for the oral administration of biopharmaceuticals has been described and validated in preclinical experiments as well as a clinical trial. More recently, we have reported that active in situ synthesis and mucosal delivery of ovalbumin (OVA) by genetically engineered L. lactis (LL) induces antigen specific oral tolerance in OVA T-cell receptor transgenic mice (DO11.10), by the induction of CD4+CD25- regulatory T cells that function through a TGF-β dependent mechanism. In the present study we further developed this approach to investigate the possible induction of antigen-specific tolerance in a well-established genotypic celiac disease mouse model. We therefore genetically engineered LL to secrete a deamidated DQ8 epitope that is immunodominant for DQ8 mediated T-cell responses (LL-eDQ8d) and subsequently studied its oral supplementation in deamidated DQ8 peptide-immunized NOD AB DQ8 MHC class II transgenic mice. NOD AB DQ8 is a mouse model that utilizes the NOD background which contributes to autoimmunity and pathogenesis in combination with a human DQ8 MHC class II transgene, which contributes to the sensitivity to gliadin. Here, we report that the active mucosal delivery of deamidated DQ8 epitopes (DQ8d) by genetically modified LL induces suppression of local and systemic DQ8 specific T cell responses in NOD AB DQ8 transgenic mice and provide a method for the induction of DQ8d antigen-specific tolerance. Moreover this approach provides a method to deliver the right antigens in an adequate manner to the intestinal mucosal immune system in the context of a non-colonizing, non pathogenic bacterium and has the potential for being an effective and non-toxic treatment of celiac disease.
Chapter 5

Methods

Bacteria and media

The genetically modified *Lactococcus lactis* MG1363 (LL) strain was used throughout this study\(^{14}\). Bacteria were cultured in GM17E medium, consisting of M17 broth (Difco Laboratories, Detroit, MI) supplemented with 0.5% glucose and 5µg/ml erythromycin (Abbott). Stock suspensions of LL 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 h of culture, a saturation density of \(2 \times 10^9\) colony forming units (CFU) per ml was reached. Bacteria were harvested by centrifugation and 10-fold concentrated in BM9 inoculation buffer at \(2 \times 10^9\) bacteria/100 µl. The dose used in all experiments was 100 µl of this suspension administered daily by intragastric catheter.

Construction of genetically modified LL-eDQ8d (Fig. 1 and Tab.1)

A genetically engineered *L. lactis* strain, designated LL-eDQ8d, was constructed to secrete an immunodominant deamidated DQ8-restricted peptide (DQ8d) (Fig. 1). The sequence encoding the deamidated DQ8 epitope was retrieved from published data\(^{15}\). In summary, two glutamine residues within the alpha-gliadin peptide were changed into glutamic acids to stimulate the deamidated immunodominant alpha-gliadin response for DQ8 carrying celiac disease patients, and this epitope is recognized by T cells of NOD ABo DQ8+ mice \(^{16}\) (Tab. 1). The DQ8d cDNA fragment was synthetically constructed (Operon, The Netherlands) and amplified by Polymerase Chain Reaction (PCR) using the following forward and reverse primers 5’caatacccatcaggtgaaggttc3’ and 5’cgactagttaagcttgtgggttttcttgtgat3’. For detection purposes an E-tag (e) was attached to the fragment (Tab. 1). To add the E-tag to the 5’ end of DQ8d gene, we used the PCR product that was produced in step 1 (DQ8d) as template in a PCR with oligonucleotides 5’ggtgctccagtccccatcagcgtgaaggttc3’ and 5’cgactagttaagcttgtgggttttcttgtgat3’. The amplified fragment was fused to the Usp45 secretion signal of the erythromycin resistant pT1NX vector, downstream of the lactococcal P1 promotorman17, 18. MG1363 strains transformed with plasmids carrying eDQ8d cDNA were designated *Lactococcus lactis* secreting eDQ8d (LL-eDQ8d). The LL-pT1NX, which is MG1363 containing the empty vector (pT1NX) served as control. The coding sequence of the circular vector was confirmed by sequence analysis and constitutive eDQ8d secretion did not alter the growth rate of *L. lactis*. 

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For functional analysis of the secreted eDQ8d epitope a proliferation assay with human T cell clones derived from the intestines of celiac disease (CD) patients was performed. Bacteria were grown overnight as described before, diluted 1:50 and grown for another 4 or 6 hours respectively. T cell clones specific for gluten were generated from a small intestinal biopsy taken from an adult Dutch CD patient that had been on a gluten-free diet for several years as described19. The patient gave informed consent to the study, which was approved by the hospital ethics committee. The patient was typed serologically to be HLA-DR3/4, DQ2/8, thus carrying both CD-associated DQ dimers. T cell clone II29 was found to respond to an alpha-gliadin derived peptide with a minimal 9 amino acid core QGSFQPSQQ, when bound to HLA-DQ819. Deamidation of the P1 and/or P9 glutamine residue (Q) into glutamic acid (E) by the activity

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**Table 1** The coding and protein sequences of the deamidated DQ8 epitope synthesized and secreted by the genetically modified LL-eDQ8d.

<table>
<thead>
<tr>
<th>Coding sequence:</th>
<th>E-tag: ggt gct cca gtt cca tac cca gat cca ctt gaa cca cgt</th>
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<td></td>
<td>DQ8d: caa tac cca tca ggt gaa ggt tca ttc caa cca tca caa gaa aac cca caa gct</td>
</tr>
<tr>
<td>Protein sequence:</td>
<td>E-tag: GAPVPYPDPLEPR</td>
</tr>
<tr>
<td></td>
<td>DQ8d: QYPSGEQSFQPSQENPQA</td>
</tr>
</tbody>
</table>

**Functional analysis of secreted epitopes**

For functional analysis of the secreted eDQ8d epitope a proliferation assay with human T cell clones derived from the intestines of celiac disease (CD) patients was performed. Bacteria were grown overnight as described before, diluted 1:50 and grown for another 4 or 6 hours respectively. T cell clones specific for gluten were generated from a small intestinal biopsy taken from an adult Dutch CD patient that had been on a gluten-free diet for several years as described19. The patient gave informed consent to the study, which was approved by the hospital ethics committee. The patient was typed serologically to be HLA-DR3/4, DQ2/8, thus carrying both CD-associated DQ dimers. T cell clone II29 was found to respond to an alpha-gliadin derived peptide with a minimal 9 amino acid core QGSFQPSQQ, when bound to HLA-DQ819. Deamidation of the P1 and/or P9 glutamine residue (Q) into glutamic acid (E) by the activity
of tissue transglutaminase was found to substantially enhance the T cell stimulatory capacity of this gluten peptide. Proliferation assays were performed in duplicate or triplicate in 150 μl culture medium (Iscoves) in 96-well flat-bottomed plates (Falcon) using 10^4 T cells stimulated with 10^5 HLA-DQ-matched and 3000 RAD irradiated Peripheral blood mononuclear cells in the absence or presence of supernatant at several concentrations. After 48 hours, cultures were pulsed with 0.5 uCi of ^3H-thymidine, harvested 18 hours thereafter upon which ^3H-thymidine incorporation was determined as a measure for proliferation.

**Mice**

Transgenic mice that express HLA-DQ8 in an endogenous MHC II-deficient background (AB^0 DQ8^+) were backcrossed to NOD mice for 10 generations and intercrossed to produce congenic NOD AB^0 DQ8^+ mice, as described previously.

NOD AB^0 DQ8 transgenic and NOD AB^0 mice were previously generated and bred at the Department of Immunology, Mayo Clinic, Rochester, MN, USA. Seven to sixteen week old mice were used for the experiments. Mice were weaned and maintained on gluten free chow and were kept in a conventional animal facility until 8-12 weeks of age. All experiments performed were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic College of Medicine.

**Antigen and Antibodies**

Deamidated DQ8 epitopes with and without E-tag (GAPVPYPDPLEPR QYPSGEGSFQPSQENPQA) were synthesized by the Mayo Proteomics Research Center (MPRC) at Mayo Clinic, Rochester. For T-cell phenotyping; CD4 and CD25 antibodies were purchased from BD-Biosciences (San Jose, CA), and APC anti-Foxp3 staining kits were purchased from eBiosciences (San Diego, USA) respectively. Anti-IL-10 neutralising monoclonal antibody (1 μg/ml, clone JES052A5), TGF-β neutralizing monoclonal antibody (1 μg/ml, clone 1D11) and LAP neutralizing antibodies (1 μg/ml, clone 27235) were obtained from R&D systems (Minneapolis, MN).

**Oral feeding and Delayed-type hypersensitivity (DTH) reaction**

NOD AB^0 DQ8 mice on a gluten free chow were sensitized by subcutaneous injection of 100 μg deamidated eDQ8 peptides in 100 μl of a 1:1 CFA (purchased from Difco of Becton, Dickinson and Company, San Jose, CA) saline solution in the tail base at day 1. The peptide used for the
sensitization had the same sequence as the secreted epitope. Mice were fed BM9 as a negative control, LL-pT1NX or LL-eDQ8d at days 1-10, dissolved in 100 µl BM9. Feedings were performed by intragastric administrations of antigen or bacterial suspensions using an 18-gauge stainless gavage needle. Ten days after immunization, antigen-specific DTH responses were assessed. Twenty-four hours thereafter DTH measurements were performed. For measurement of antigen-specific DTH responses, baseline ear-thickness was measured using an engineer’s micrometer (Mitutoyo, Tokyo, Japan). Mice were then injected with 10 µg eDQ8d in 10 µl saline in the auricle of the ear. The ear-thickness was measured again in a blinded fashion at 24 h after challenge. DTH responses were expressed as the difference in the baseline ear-thickness and the ear-thickness 24 hours after eDQ8d injection. Subsequently mice were sacrificed, spleen and lymph nodes were harvested and cells were assessed for DQ8d-specific proliferation and cytokine production. To test for E-tag interference NOD AB° DQ8 mice were immunized with 100 µg deamidated DQ8 peptides with (eDQ8d) or without e-tag (DQ8d) in 100 µl of a 1:1 Complete Freund’s Adjuvant (CFA, Difco, BD) saline solution in the tail base at day 1. At day 7 mouse DTH measurements were performed as described above with 10 µg DQ8d with or without E-tag, corresponding to the peptide used for the immunization.

Cell cultures, proliferation and cytokine production assays

Cell suspensions of spleen and lymph nodes were prepared at day 11 of the experiment by homogenizing the tissue with a tissue grinder (VWR International, Inc, North Mankato, MN) in 1X PBS. Erythrocytes were removed from the spleen cell suspensions by incubation with Ammonium Chloride/Potassium lysis buffer. Cells were incubated in 96-well microtiter plates at 5x10^5 cells/well in 0.2-ml volumes at 37°C in RPMI 1640 (1.5% Hepes, 1% Penstrep and 10% FBS) with supplements containing either medium alone, 10 µg Con A, or 50 µg eDQ8d epitope. In a separate experiment IL-10, TGF-β, IL10&TGF-β or LAP neutralizing antibodies were added to splenocytes of LL-eDQ8d treated mice. After 24 h, proliferation was assessed by addition of 1 µCi/well [3H]-thymidine for the last 24 h of culture. DNA-bound radioactivity was harvested onto glass fiber filter mats (Perkin Elmer, Boston, USA) and thymidine-incorporation measured on a scintillation counter (Perkin Elmer). Results were expressed as mean cpm of triplicate wells. For cytokine measurements, supernatants of the cell cultures used in the different proliferation assays, described above, were collected after 24 h of culture and frozen at –20° C until cytokine analysis was performed. Cytokine production was quantified using the
Mouse Inflammation Cytometric Bead Assay (BD Biosciences, Mountain View, CA, USA).

**Flow cytometric analysis**

Spleens and gut-associated lymph node tissue (GALT) of BM9, LL-pT1NX or LL-eDQ8d treated mice were isolated, prepared as described above and stained for CD4, CD25 and Foxp3. Intracellular staining was performed for Foxp3 according to the manufacturer’s instructions (eBiosciences, San Diego, CA) and subsequently measured using flow cytometry on a Becton Dickinson FACSCaliburs (Mayo Clinic College of Medicine Flow Cytometry/Optical Morphology Core Facility). Cells were gated on CD4+CD25+ and CD4+CD25- subpopulations and within these populations Foxp3 histograms were used to determine Mean Fluorescence Intensity (MFI).

**Statistical analysis**

Results from cytokine measurements are expressed as mean ± SEM. eDQ8d-specific proliferation, ear-thickness, and cytokine measurements were tested for significance using one-way ANOVA followed by the student’s t-test comparison to determine the differences between individual groups. For all tests a p value <0.05: *, <0.01: ** was used to indicate statistical significance for both tests.

**Results**

**Synthesis and secretion of functional deamidated DQ8 epitopes (Figure 2a and b)**

*In vitro* synthesis and functionality of the secreted deamidated DQ8 epitopes was confirmed by a proliferation assay with human DQ8 T cell clones derived from the intestine of celiac disease (CD) patients. T cell clones derived from the intestine of celiac patients were stimulated with supernatant of a LL-eDQ8d culture at different concentrations (Fig. 2a). Control (LL-pT1NX) supernatant did not induce proliferation (data not shown). The secreted immunodominant deamidated DQ8 peptide contains an amino-terminal E-tag for detection purposes. To exclude a possible interference of the E-tag with the functional properties of the peptide, NOD AB° DQ8 transgenic mice were immunized with DQ8d epitopes with or without E-tag at day 0. At day 7, DTH response measurements were performed with the injection of 10 µg DQ8d epitope. The presence of an
E-tag did not change the DTH response in DQ8d immunized mice (Fig. 2b). Immediately after the DTH measurement, bulk splenocytes and bulk inguinal lymph node cells were isolated and restimulated ex vivo with DQ8d epitopes with or without E-tag. Again, the presence of the E-tag did not change the immune-stimulating properties of the DQ8d epitope (data not shown). These data demonstrate that *L. lactis*-derived eDQ8d is fully bioactive and that the addition of the E-tag does not interfere with its functionality.

**Suppression of the DQ8d-induced DTH and peripheral immune response by mucosal delivery of eDQ8d epitopes by *L. lactis*** *(Figure 3)*

We subsequently investigated the effect of oral administration of LL-eDQ8d on the eDQ8d-induced DTH. eDQ8d-immunized NOD AB° DQ8 transgenic mice were fed BM9 (inoculation buffer, as a negative
control), LL-pT1NX (empty vector control) or LL-eDQ8d intra-gastrically for 10 consecutive days. On day 10, mouse ears were injected with 10 μg eDQ8d and 24 hours later ear-thickness measurements were performed. Control mice (fed BM9) were clearly immunized to eDQ8d, but daily intra-gastric administration of LL-eDQ8d significantly reduced the DTH response (13.1x10^{-2} mm vs 5.1x10^{-2} mm, p=0.0031) (Fig. 3a). Ear swelling was also slightly reduced in LL-pT1NX-treated mice compared to controls (9.3x10^{-2} mm vs 13.1x10^{-2} mm p=0.0343) but to a much lesser degree than in LL-eDQ8d treated mice. NOD AB° mice (without DQ8 transgene) showed

Figure 3 Mucosal delivery of eDQ8d epitopes by *L. lactis* significantly decreases the DQ8d-induced DTH response and proliferative capacity of bulk spleen and inguinal lymph node cells.

NOD AB° DQ8 transgenic mice were immunized by s.c. injection of 100 μg eDQ8d in CFA at day 1. Mice were orally treated with LL-eDQ8d or LL-pT1NX at days 1-10. Control mice received BM9. At day 10, mice were challenged with 10 μg eDQ8d in 10 μl saline in the auricle of the ear. DTH responses are expressed as the mean (±SEM) increase in ear thickness from baseline, 24 hours after injection (a). After the DTH measurements, spleens and inguinal lymph nodes of the BM9 (control), LL-pT1NX and LL-eDQ8d groups were isolated and *ex vivo* restimulated with 50 μg eDQ8d peptide. eDQ8d-specific proliferative response of bulk splenocytes (p=0.048) (b) and inguinal lymph node cells (p=0.002) (c) were studied by Thymidine incorporation, expressed as the mean (±SEM) cpm. Results summarize data of 3 independent experiments including 6 mice per group.
only a minor increase in ear thickness (3.2x10^{-2} mm). These data indicate that orally administered LL-eDQ8d suppresses systemic inflammatory T-cell responses in immunized NOD AB^o DQ8 transgenic mice and that the secreted antigen is necessary for induction of a significant tolerogenic effect.

Peripheral immune responses were further analyzed by investigating eDQ8d-specific proliferation of spleen and draining inguinal lymph node cells (ILN). Splenocytes of mice treated with BM9, LL-pT1NX or LL-eDQ8d were isolated on day 11 after immunization, and the eDQ8d-specific proliferative response was assessed by ex vivo stimulation with eDQ8d peptides. Splenocytes of immunized mice showed a high eDQ8d-specific proliferative response that was significantly suppressed by daily intra-gastric administration of LL-eDQ8d (13.1x10^3 vs 5.6x10^3 CPM, p=0.048) (Fig. 3b). Since the inguinal lymph nodes are the primary antigen recognition site in this immunization protocol, we also examined the proliferative capacity of these lymphocytes. Proliferation of inguinal lymph node cells was much decreased in the LL-eDQ8d treated group compared to the BM9 treated group (1.4 x10^3 vs 2.8x10^3 CPM, p= 0.002) (Fig. 3c). These data indicate that LL-eDQ8d treatment is able to suppress systemic T cell responses in NOD AB^o DQ8 transgenic mice.

Decrease in inflammatory cytokines and increase in IL-10 production after LL-eDQ8d treatment. (Figure 4)

To investigate the mechanisms behind the reduction of antigen-induced T cell proliferation, we determined cytokine profiles of ex vivo stimulated splenocytes or inguinal lymph node cells. Ex vivo eDQ8d stimulated spleen cells showed a significant up-regulation of IL-10 (52.6 vs 5.4 pg/ml, p= 0.0022) and a downregulation of IL-12 production (8.0 vs 27.6 pg/ml) only in the LL-eDQ8d treated group compared to the negative control (BM9) (Fig. 4a). Moreover LL-eDQ8d treatment significantly reduced the eDQ8d-induced IFN-γ production (380 vs 1328 pg/ml, p=0.0087) in the inguinal lymph nodes compared to the negative control (BM9) treated mice (Fig 4b). Together, these data indicate that the reduced proliferative capacity of the spleen and ILN cells of the LL-eDQ8d treated mice is accompanied by decreased inflammatory cytokine production and increased IL-10 production.
The functional importance of TGF-β, IL-10, and LAP (membrane-associated TGF-β) for the eDQ8d-specific splenic proliferative response of splenocytes from LL-eDQ8d treated mice was assessed using neutralizing antibodies. The individual neutralization of IL-10-, TGF-β- or LAP did not significantly interfere with the decreased splenic proliferative response of LL-eDQ8d treated mice, but adding a combination of TGF-β and IL-10 suppressed the response. This suggests that both TGF-β and IL-10 are essential for LL-eDQ8d mediated suppression.

**Figure 4** LL-eDQ8d treatment decreases inflammatory cytokine production and increases IL-10 production. Cytokine measurements in the supernatant of splenocytes (a) and inguinal lymph node cells (b) were performed 24 hours after *ex vivo* eDQ8d stimulation. Results represent the mean (±SEM) of cytokine secretion in pg/ml for at least two individual experiments including 6 mice in each group.

**Critical role for both TGF-β and IL-10 in LL-eDQ8d mediated suppression (Figure 5)**

The functional importance of TGF-β, IL-10, and LAP (membrane-associated TGF-β) for the eDQ8d-specific splenic proliferative response of splenocytes from LL-eDQ8d treated mice was assessed using neutralizing antibodies. The individual neutralization of IL-10-, TGF-β- or LAP did not significantly interfere with the decreased splenic proliferative response of LL-eDQ8d treated mice, but adding a combination of TGF-β and IL-10 suppressed the response. This suggests that both TGF-β and IL-10 are essential for LL-eDQ8d mediated suppression.

**Figure 5** Decreased splenic eDQ8d-specific proliferation depends on IL-10 and TGF-β. Mice were fed BM9, LL-pT1NX and LL-eDQ8d and DTH measurements were performed as described above. We next investigated the functional importance of cytokines such as TGF-β, IL-10, TGF-β in combination with IL-10 and LAP on the eDQ8d-specific splenic proliferative response using neutralizing antibodies. To do so, bulk spleen cells of LL-eDQ8d treated mice were isolated and 5 x 10⁵ cells were stimulated *ex vivo* with 50 µg eDQ8d with or without neutralizing antibodies. Proliferative responses are expressed as mean (±SEM) cpm. Results are representative of two individual experiments with 6 mice each per group.
neutralizing monoclonal antibodies completely abolished the decreased eDQ8d-specific proliferative capacity of splenocytes (4.9x10^3 vs 1.6x10^3 CPM, p= 0.0097) (Fig. 5). These data strongly suggest that the T cell activation suppression mediated by LL-eDQ8d treatment is dependent upon an interplay between IL-10 and TGF-β.

**Increase in Foxp3 expression by CD4^+CD25^+ and CD4^+CD25^- T cells. (Figure 6)**

To analyze the role of regulatory T cells (Treg) in the induction of LL-eDQ8d-induced tolerance, we investigated the expression of Foxp3 within the CD4^+ T cell population by FACS analysis. A significant upregulation of Foxp3 was seen within the CD4^+CD25^+ as well as the CD4^+CD25^- splenic cell population of LL-eDQ8d treated mice compared to the control group (BM9) (MFI 171 vs. 61 and 35 vs. 6, respectively) (Fig 6a and 6b). Foxp3 was also upregulated in the CD4^+CD25^- population in the gut-associated lymph node tissue (GALT) of the LL-eDQ8d treated mice compared to the BM9 treated (MFI in M1 73 vs. 30) (Fig 6c), but not in the GALT CD4^+CD25^+ population. LL-pT1NX feeding also induced some Foxp3 upregulation, but exclusively in the splenic CD4^+CD25^- T-cell population and to a lesser extent than LL-eDQ8d (MFI 15 vs. 35, respectively).

**Figure 6** LL-eDQ8d treatment significantly increases splenic and GALT Foxp3 expression. At day 11, spleens and gut associated lymph node tissue (GALT) of mice treated with BM9 (blue), LL-pT1NX (yellow) or LL-eDQ8d (pink) were isolated and stained for CD4, CD25 and intracellular Foxp3. Flow cytometry was performed on the splenic CD4^+CD25^+ (a), CD4^+CD25^- (b) and GALT CD4^+CD25^- (c) subpopulations. Data represent 1 experiment with 6 mice per group.
Chapter 5

Discussion

Our data demonstrate that genetically modified *L. lactis* can be used for mucosal delivery of functional immunodominant antigens, and that this approach suppresses inflammatory antigen-specific T cell responses in gliadin-sensitized NOD AB0 DQ8 transgenic mice. Furthermore, our results suggest that the induced suppression is mediated by Foxp3+ CD4+CD25+ and CD25- regulatory T cells that possibly function through an IL-10 and TGF-β dependent mechanism.

Successful clinical application of antigen-specific mucosal tolerance for the treatment of human diseases has been difficult to achieve and critically depends on several factors, including the purity, source, dose and the mode of antigen presentation to the mucosal immune system. Several protocols for induction of oral tolerance, including oral administration of the antigen with IL-10, have been shown to induce antigen-specific Tr1 cells that suppress undesired immune responses toward self-antigens, allergens, and food antigens. Although a previous clinical attempt to induce tolerance in celiac disease by the administration of rhIL-10 in refractory celiac disease patients was ineffective, strategies to boost the number and/or function of Ag-specific Tr-1 cells may offer new therapeutic opportunities. This notion is supported by the finding that gliadin-specific mucosal regulatory T cells from celiac disease patients are able to suppress proliferation of pathogenic Th0 cells. 

Both dendritic cells (DC) and Treg are critically involved in tolerance induction. We recently demonstrated that exposure to *L. lactis* alters DC phenotype and 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 hypothesize that induction of antigen-specific regulatory T cells in our experiments was mediated by altered presentation of the immunodominant peptide by dendritic cells, and our observation that LL-eDQ8d treatment interfered with IL-12 production of splenocytes, suggests that at least part of the tolerogenic effect is DC-mediated. However, it should be noted that the spleen is not the primary antigen-recognition site in our model, and alternatively, activation of regulatory T cells may have resulted in a reduced activation of antigen-presenting cells.

We further demonstrated that LL-eDQ8d treatment reduced peripheral DTH responses as well as eDQ8d-specific proliferation of bulk splenocytes and inguinal lymph node cells. LL-pT1NX treatment also somewhat reduced the DTH and splenic proliferative capacity but less pronounced than the LL-eDQ8d treated mice. The LL-eDQ8d treatment-mediated IL-10 secretion and the reduction of IL-12 and IFN-γ production that was
found after ex vivo stimulation (of splenocytes) was not observed in the LL-pT1NX treated mice. These data confirm our previous findings that the tolerogenic effect, at least in part is *L. lactis* mediated but that the co-delivery of low-dose antigen, in this case deamidated DQ8 peptide, greatly enhances the induction of antigen-specific oral tolerance.

In recent years it has become apparent that Treg play a critical role in the induction and maintenance of oral tolerance\(^{29, 30}\). Still many questions need to be answered concerning the phenotype and complexity of Treg as well as the precise role and different overlaps in oral tolerance.

Several phenotypically and functionally distinct Treg subsets have been implicated in suppression of intestinal inflammation and induction of oral tolerance, including adaptive Treg (aTreg), comprising Th3 and Tr1 cells, and naturally occurring Treg (nTreg), which maintain tolerance to self-antigen under normal physiological conditions. Although it is probable that nTreg play a central role in regulating gut immune homeostasis, their precise function remains to be characterized\(^{5, 31, 32}\). Furthermore, recently a separate category of Treg has been described that acquires *Foxp3* upon TGF-\(\beta\) stimulation. These so-called inducible Treg (iTreg) have regulatory functions both *in vitro* and *in vivo*\(^{33, 34}\). This recently discovered subset mimics the Treg induced in this model, as both subsets are induced in the periphery, express *Foxp3* and are critically dependent on TGF-\(\beta\)/IL-10.

To map the Treg that mediated oral tolerance in our experiments, we studied the functional importance of TGF-\(\beta\), IL-10, and LAP (membrane-associated TGF-\(\beta\)) on the eDQ8d-specific splenic proliferative response using neutralizing antibodies. Interestingly only the combined neutralization of IL-10 and TGF-\(\beta\) interfered with the proliferative capacity of the splenocytes suggesting an interactive role for both anti-inflammatory cytokines. The exact mechanism by which these cytokines function is not completely understood but both cytokines frequently interact during regulatory Treg responses\(^{35-37}\).

Furthermore we found a significant *Foxp3* upregulation in both the mucosal and the splenic CD4\(^+\) T-cell population. It is known that antigen-specific TGF-\(\beta\) producing Th3 cells drive the differentiation of antigen-specific *Foxp3*\(^+\) regulatory cells in the periphery\(^{38}\). Furthermore TGF-\(\beta\)-dependent conversion of peripheral CD4\(^+\)CD25\(^{-}\) T cells into CD25\(^+\), CD45RB\(^{-}/\text{low}\) suppressor cells has been reported\(^{33}\). We have not studied the origin of the *Foxp3*\(^+\) Treg in detail, but our data suggest that either mucosal ‘adaptive’ CD4\(^+\)CD25\(^{-}\) Treg were induced by LL-eDQ8d treatment which eventually converted into CD4\(^+\)CD25\(^+\) Treg or that the induced Treg comprise a separate CD4\(^+\)CD25\(^{\text{low}}\) lineage\(^{39}\). Previously it has been
shown that oral tolerance induced by CTB-conjugated Ag is associated with increased TGF-β production and the generation of Foxp3+CD25+CD4+ and both Foxp3+ and Foxp3−CD25−CD4+ Treg. The relationship between thymus derived natural CD4+CD25+Foxp3+ Treg and other subpopulations induced in the periphery, e.g., peripherally generated CD4+ Foxp3+ cells, as well as Tr1 and Th3 cells needs clarification, but these data suggest a significant overlap and interactive function in the induction of mucosal tolerance.

Direct in vivo induction of tolerogenic DC or Treg is a major target for immunotherapy for allergic, autoimmune and several inflammatory diseases and can be achieved by exposing the mucosal immune system to low doses of antigen. We here report that oral supplementation of a genetically modified L. lactis secreting deamidated DQ8 peptides greatly reduces systemic immune responses induced by that antigen in DQ8d-immunized NOD ABo DQ8 transgenic mice. The suppression is mediated by the induction of Foxp3+ Treg that are dependent on both TGF-β and IL-10. These observations further support the development of gut delivered bacteria enhanced oral tolerance for the treatment of both mucosal and systemic autoimmune, inflammatory or allergic diseases by specific antigen-secreting L. lactis and can be rapidly translated into a therapy for celiac disease.

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Footnotes

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Chapter 5


