Antigen-specific oral tolerance for the treatment of inflammatory and allergic diseases
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Citation for published version (APA):
Summary and general discussion

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Chapter 9

Summary and general discussion

(Clinical translation of bacterial induced tolerance)

Several common inflammatory, autoimmune and allergic diseases of the gastro-intestinal tract, including celiac disease, Crohn’s disease and food allergies result from an inappropriate immune response to exogenous antigens. These diseases are associated with considerable morbidity and their current therapies, consisting of dietary antigen exclusion or general immune suppression, are often ineffective, do not modify the natural course of the disease and are associated with several unwanted effects. These include a significant decrease of the quality of life in case of food avoidance and serious short and long-term side effects resulting from immunosuppressive therapies. Therefore there is an unmet need for novel therapeutic approaches and because these diseases are initiated by aberrant immune responses to certain proteins, the Holy Grail would be the induction of oral tolerance (either antigen-specific or through bystander suppression). The advantages of this approach are a lack of toxicity, ease of administration over time and antigen-specific mechanisms of action. The efficacy of 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 tolerance for therapeutic purposes in humans have failed. Nonetheless, these previous attempts, using proteins or peptides have identified several factors that are critical for induction of oral tolerance, including dose, route of administration (nasal vs oral vs transcutaneous), formulation, application of mucosal adjuvant, and the timing of therapy. Moreover previous experimental data indicate that heterogeneous antigen mixtures are less effective inducers of oral tolerance than single purified antigens. It is commonly accepted that oral tolerance can results from two major mechanisms: Administration of a low antigen dose favors active suppression, whereas higher doses favor clonal anergy or deletion. When anergy or clonal deletion is desired, 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 and knowledge of the causal antigen is not required. The aim of the work summarized in this thesis was to develop clinically applicable methods for induction of oral tolerance, which is defined as induction of suppression of immune responses to an antigen by oral administration.
It is well known that induction of oral tolerance using proteins or peptides in the experimental setting depends on the purity, source, dose of antigen and the mode of antigen presentation to the mucosal immune system. In this thesis, we propose a novel therapeutic strategy for the induction of antigen specific oral tolerance by the active intestinal synthesis and delivery of an antigen using genetically engineered \textit{L. lactis}. This approach obviates the need for large scale purification of human (auto)antigens and circumvents current issues related to induction of oral tolerance in humans. Here, we demonstrate that mucosal delivery of an antigen by genetically modified \textit{L. lactis} induces suppression of local and systemic antigen-specific T-cell responses in two different transgenic mouse models, comprising DO11.10 and NOD AB\(^{0}\) DQ8 mice. We furthermore show that this effect is dependent on the secreted antigen but also on yet undefined characteristics of \textit{L. lactis}. Importantly, the used technology resulted in the induction of systemic tolerance probably mediated by the induction of tolerogenic dendritic cells (DC) and was associated with the local and systemic appearance of antigen-specific regulatory T cells (Treg).

The approach described in this thesis has several characteristics that may make it an attractive antigen delivery system for the use in modulating immune responses in humans. \textit{L. lactis} is a non-pathogenic, non-invasive, noncolonizing gram-positive bacterium and is Generally Regarded As Safe (GRAS) according to the US Food and Drug Administration. This bacterial strain has much homology with several probiotics. Previously, several genetically modified \textit{L. lactis} strains have been generated for local synthesis and delivery of immunomodulatory proteins to the intestinal mucosa. For example a strain of \textit{L. lactis} modified to express IL-10, the anti-inflammatory cytokine which plays a very important role in the regulation of mucosal immunity, was effective at preventing colitis in IL-10 knockout mice and after oral administration of dextran sodium sulfate. Moreover a biologically contained \textit{L. lactis} strain secreting human IL-10 was approved and used in a phase I, open label clinical trial on Crohn’s disease patients. 10 patients with moderate to severe Crohn’s disease were treated for 8 days in a placebo uncontrolled trial. Although the main goal of the study was to determine the toxicity in an uncontrolled study, several patients showed a decrease in Crohn’s disease activity index and a reduction of the CRP serum levels. This trial demonstrated that treatment of humans with viable \textit{L. lactis} secreting IL-10 is clinically and biologically safe and consequently oral administration of genetically modified \textit{L. lactis} for intestinal delivery of proteins is a clinically feasible strategy and warrant a phase 2 clinical trial. All these findings were the
foundation for the establishment of Actogenix, a commercial spin-off from VIB and Ghent University, which exploits the *L. lactis* delivery system for oral administration of biopharmaceuticals. The Company is developing a broad and diverse portfolio of novel therapeutic products addressing major diseases with high unmet medical need, which accelerates the clinical translation.

The precise mechanism by which the genetically modified *L. lactis* enhances tolerogenic signals remains unclear, but it is possible that OVA peptide and/or lactococcal antigens and/or whole *L. lactis* are taken up by M-cells that are located in Peyer’s patches or that they were directly sampled by intraluminal extensions of mucosal dendritic cells. Indeed, it has been recently reported that immune responses can be mediated through direct luminal antigen sampling by submucosal DC that protrude extensions into the gut lumen without comprising the epithelial barrier function. Interestingly, most viable bacteria and highest amounts of mucosal OVA were present in the distal small intestine (*Chapter 3*), which is also the predominant location of the intestinal sampling DC network. In addition, *L. lactis* might serve as a bio adhesive delivery vehicle that localizes the antigen delivery at the intestinal sampling network and/or that intensifies contact with the mucosa, hereby increasing the antigen concentration gradient and ensuring immediate absorption without dilution or degradation in the luminal fluid. Moreover, a bacterial-delivered antigen may provide a more effective dose of antigen or localization of the antigen presentation to the intestine than obtained with soluble oral antigen alone.

In recent years it has become apparent that DC and regulatory T lymphocytes (Treg) play a primary role in oral tolerance. The exact phenotype and functional properties of tolerogenic DC still needs to be determined, but the tolerogenic function of DC appears to involve various mechanisms including deficiency of, or signalling through, costimulatory molecules, secretion of immunosuppressive cytokines (IL-10 and TGF-β) and enzyme induction. Previously, we have demonstrated that exposure to *L. lactis* alters DC phenotype and function, which in the presence of simultaneous exposure to a DC-presented antigen may result in the generation of an antigen-specific Treg subset (*Chapter 2*). Furthermore we show that LL-OVA alters the phenotype of (mature) DC in DO11.10 mice, leading to decreased antigen presenting capacity of CD11c+ dendritic cells (*Chapter 3*). Together, these data suggest that the tolerogenic effect is, at least in part, DC mediated.
Summary and general discussion

Many questions remain to be answered concerning the phenotype and complexity of Treg as well as their precise role in induction and maintenance of 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 characterized by the secretion of the anti-inflammatory cytokines TGF-β and IL-10 respectively, and naturally occurring Treg (nTreg) which maintain tolerance to self-antigen under normal physiological conditions and are characterised by the expression of the intra-cellular transcription factor Foxp3. nTreg, next to the adaptive Treg, play a central role in regulating gut homeostasis, but their relative importance as well as functional inter-relationships remains largely unknown. A new category of Treg has been described apart from the aTreg and the nTreg that acquires Foxp3 upon TGF-β stimulation. These so-called inducible Treg (iTreg) have regulatory functions both in vitro and in vivo.

In this thesis we demonstrate that active in situ synthesis and mucosal delivery of OVA by the genetically engineered L. lactis induces antigen specific oral tolerance in DO11.10 mice, by the induction of CD4+CD25− regulatory T cells that function through a TGF-β dependent mechanism (Chapter 3) and that TGF-β neutralizing peptide (p17) completely abolished LL-OVA induced antigen specific oral tolerance in vivo in DO11.10 mice (Chapter 4). Interestingly the CD4+CD25− cell population was able to adoptively transfer tolerance in sensitized DO11.10 mice and showed a significant upregulation of Foxp3 and CTLA-4, suggesting a phenotype consistent with iTreg. Moreover IL-10 was significantly up-regulated in the cervical lymph nodes, GALT and spleen of LL-OVA treated mice, and although not required for suppression, this cytokine may be important for the generation/differentiation of the CD4+CD25−Foxp3+ cell population. These data underscore the indispensable role of Treg in this model and it would be interesting to unravel the exact phenotype, function and interaction between the different Treg subsets by FACS cell sort and its consequent functional analysis in vitro and in vivo.

After the successful application of LL-OVA in DO11.10 mice, we adapted the strategy to test the induction of antigen specific oral tolerance in NOD AB8 DQ8 class II transgenic mice, a well-established genotypical celiac disease mouse model. We report that oral supplementation of LL-eDQ8d induces suppression of local and systemic DQ8 restricted T-cell responses in NOD AB8 DQ8 class II 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 combination of IL-10 and TGF-β and associated with a significant induction of Foxp3+ regulatory T-cells (Chapter 5). We have not studied the origin of the induced Foxp3+ Treg in detail, but it is possible that 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+CD25low lineage. These data highlight how different Treg populations can overlap in function and phenotype and hint at the complexity of the regulatory pathways involved in oral tolerance. Furthermore these data indicate that L. lactis can condition the mucosal immune system toward tolerance induction and boost antigen-specific induction of Treg by the co-delivered antigen.

Different studies have demonstrated the efficacy of L. lactis treatment to abrogate Th2-type responses induced in airway hyperreactivity or food allergy murine models using Balb/c mice. However, in these studies the Th2 hypersensitivity responses were diminished by the induction of counterregulatory Th1 immune responses induced by the L. lactis. In fact oral pre-treatment 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 in murine models. It is important to realize that laboratory mice (that are not fed with cheese or dairy products) are never exposed to L. lactis, and recognize the organism as “foreign”. Interestingly, the counterregulatory Th1 effect was abrogated and tolerance was induced in longer treatment/feeding regimes and when L. lactis conditioned mice (L. lactis exposure early in life) are used in Balb/c mice, demonstrating that the Th1 adjuvant effect can be attributed to the absence of these bacteria in the mice diet (Chapter 6). 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 GRAS status, and, thus, it is acceptable to believe that L. lactis will not have any Th1 adjuvant effect in humans, although care has to be taken when extrapolating counterregulatory immune responses induced by L. lactis in mice for human applications. On the other hand, this knowledge provides promise for the use of L. lactis as oral delivery vehicle for the induction of tolerance.

Therapeutic induction of tolerogenic DC and/or Treg is a promising strategy for treating or restoring tolerance in patients suffering from autoimmune, inflammatory or allergic diseases. In (chapter 7) we review data pertaining to the existence and functional activity of regulatory T cells in
the intestinal mucosa and consider the potential therapeutic application of regulatory T cells in IBD. 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. Several challenges must be faced in the translating the information gained from mouse studies to human studies. First there is a need for a precise characterization of the (cellular) pathways that mediate the therapeutic effect and a validation of markers that can be used to demonstrate induction of tolerance in the clinical setting. Secondly, given differences in tolerance induction and functions of tolerogenic cells from different species, many questions remain unanswered until phase I and phase II clinical trials have been conducted. Thirdly, clinical trials need to determine dose-responses of the antigen as well as the bacteria. The advantage of the LL antigen delivery system is the direct in vivo induction of potent regulatory T cells by targeting antigens to DC by the mucosal route. Engineered L. lactis circumvents several current problems related to the induction of oral tolerance in human and could be an effective tool for inducing antigen-specific tolerance, with possible application in the treatment of antigen-induced autoimmune, allergic and inflammatory diseases.

In conclusion (chapter 9 and 10), this thesis provide important data that L. lactis can be used to efficiently deliver antigen to the intestinal mucosa for the induction of antigen-specific peripheral tolerance. This mode of administration induces much more efficient responses than with purified antigen and alleviates the need for large scale protein purification. Adequate biological containment strategies and efficient antigen delivery make L. lactis an attractive candidate for tolerance induction to known antigens. This approach gives hope for novel therapeutic interventions in antigen-driven diseases such as allergies or some autoimmune diseases and can be rapidly translated into a therapy for the treatment of both mucosal and systemic autoimmune, inflammatory and/or allergic diseases by specific antigen-secreting L. lactis.