Antigen-specific oral tolerance for the treatment of inflammatory and allergic diseases
Huibregtse, I.L.

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General introduction

Huibregtse IL\textsuperscript{1}, de Jong EC\textsuperscript{2}, van Deventer SJH\textsuperscript{3}

\textsuperscript{1} Center for Experimental and Molecular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands
\textsuperscript{2} Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands
\textsuperscript{3} Department of Gastroenterology, Leids Universitair Medisch Centrum, Leiden, the Netherlands
Chapter 1

**General introduction**

The human gastrointestinal tract comprises an enormous mucosal surface area (approximately 200-400 m²), which is continuously exposed to a variety of foreign antigens, such as food proteins, commensal bacteria and pathogens. The mucosal immune system is equipped to discriminate between harmless antigens and antigens expressed by pathogens which induce very different reactions. Harmless agents such as dietary antigens, commensal enteric bacteria and most intestinal antigens induce immunologic hyporesponsiveness or tolerance, whereas recognition of pathogens causes an active non-tolerant inflammatory response. Because both the presence of commensal bacteria in the intestinal tract and the uptake of nutrients are essential for normal development, oral tolerance is essential for life¹. Several autoimmune, inflammatory and allergic diseases of the gastrointestinal tract are a result of failure to induce, or a breakdown of normal mucosal tolerance. In this introduction we will shortly describe the mechanism of oral tolerance, the most important cell types involved -comprising tolerogenic dendritic cells and regulatory T cells- and the induction of oral tolerance as a possible therapy for several common autoimmune inflammatory and allergic diseases.

**Oral tolerance**

The induction of tolerance to dietary proteins and commensal bacteria represents the major immunological event taking place in the gut in physiological conditions. The classical textbook definition is the specific suppression of cellular and/or humoral immune responses to an antigen induced by its prior administration by the oral route. Because many of the antigens involved are only encountered following the establishment of central tolerance within the thymus, oral tolerance is a state of active inhibition of antigen-specific immune responses. Although the mechanisms by which tolerance is induced still need to be fully characterized it is generally accepted that there are two primary effector mechanisms: the induction of regulatory T cells that mediate active immune suppression and the induction of clonal anergy (functional unresponsiveness) or deletion (programmed cell death). An important factor that determines the form of peripheral tolerance induced is antigen dose. Low-dose antigen administration favors the generation of regulatory cell-driven tolerance (e.g. antigen-specific regulatory T cells expressing suppressive factors), whereas high-dose antigen administration favors clonal deletion or anergy of the T cells recognizing the antigen², ³. The different mechanisms of tolerance induction are not mutually exclusive
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and may overlap. Multiple effector mechanisms of tolerance are induced by oral antigen administration, comprising Th2 cells producing IL-4 and IL-10, Th3 cells producing TGF-β, CD4+CD25+ regulatory T cells and latency-associated peptide+ T cells11 of which the exact mechanism still needs to be determined. Several factors have been identified that enhance oral tolerance in an experimental setting, such as IL-4, IL-10, anti IL-12, TGF-β, cholera toxin B subunit, and anti-CD40 ligand12-16.

The gastro-intestinal mucosal immune system

The gut associated lymphoid tissue (GALT) is the largest immunologic organ in the body and controls a complex array of innate and adaptive mechanisms of immunity17. The GALT consists of organized lymphoid structures such as Peyer’s Patches (PP) which are large aggregates of lymphoid tissue found in the small intestine18, mesenterial lymph nodes (MLN), isolated lymphoid follicles (distributed throughout the wall of the intestines)19, and isolated immune cells, predominantly lymphocytes, scattered throughout the epithelium and lamina propria20. The stimulation of cells in the GALT by intestinal antigens can result either in immunity or tolerance to that antigen and involves APC-T cell, T cell-T cell, and T cell-B cell interactions as have been observed in other lymphoid tissues. The exact factors that determine the decision between tolerance or inflammation are not completely understood, but are most likely a result of cellular interactions within the GALT. Peyers patches are not an absolute requirement for the induction of either high- or low-dose oral tolerance, although mice lacking as well the MLN as the PP are refractory to the induction of oral tolerance21-23. Although the exact route of antigen uptake and presentation within the mucosa-draining lymphoid tissue remains unclear, these data emphasize the critical role of the gut-draining MLN in the induction of a tolerogenic mucosal immune response after oral antigen application.

An important factor that needs to be considered when studying mucosal immune responses is the barrier composed of the single layer of gut epithelial cells. Access of intact antigens to the epithelium is limited, because of a tissue barrier formed by tight junctions and directed degradation (digestion) of proteins by cellular enzymes (proteolysis) and acid secretion20. Nonetheless a significant antigen load is capable of entry into the mucosa. A next line of defence is formed by the innate immune system which refers to immediate defense against infection by nonspecific mechanisms and functions through various effector mechanisms, comprising various innate defence cells (dendritic cells, neutrophils,
monocytes, macrophages and NK cells), cells that release inflammatory mediators (basophils, mast cells, and eosinophils) and molecules (such as complement proteins, acute phase proteins, and cytokines). The cells of the innate immune system recognize, and respond to, pathogens in a generic way and most have a unique capacity to instruct the adaptive arm of the immune system, which refers to the antigen-specific immune response which confers long-lasting or protective immunity.

**Antigen processing (figure 1)**

Gut luminal antigens are taken up and presented by various routes. Specialized M-cells (or microfold cells) are traditionally thought to represent the main entry site for antigen uptake in the small intestine. These cells are found in the follicle associated epithelium lining the PP and have the unique ability to sample antigen from the lumen of the small intestine and then pass the antigen to dendritic cells. Via M-cells, some local dendritic cells (DC) prime T cells within the interfolliculair area of the PP, but most of the dendritic cells migrate to the local MLN to prime naïve CD4+ T cells. Specialized submucosal DC located in the gut epithelium have the capacity to directly sample antigens without comprising the epithelial barrier function by direct luminal sampling. This recently identified DC sampling network is predominantly located in the distal small intestine. Following antigen uptake, these DC migrate to the MLN to prime naïve CD4+ T cells. In the lamina propria antigens can also locally be presented to T cells by MHC-II expressing enterocytes or professional antigen-presenting cells (APC), such as dendritic cells. However, because naive CD4+ T cells are rare in the lamina propria, most antigen-loaded APC migrate out of the gut mucosa or PP via the afferent lymph to the MLN where they are able to instruct naïve CD4+ T cells in the specialized T cell zones. Instructed T cells leave the MLN via the efferent lymph and after entering the blood stream migrate either to the mucosa to induce local immune responses or to the periphery for the induction of systemic immune responses. Furthermore particularly in the case of high-dose antigen exposure, free antigens may reach the MLN via the afferent lymph without being carried by epithelial DC and will be presented to naïve T cells by local MLN DC. Besides local antigen presentation, free antigens might also gain direct entrance to the blood stream from the gut, via the liver and thereafter interact with T cells in peripheral lymphoid tissues such as the spleen. The route, the type and the context by which an antigen is presented to and taken-up by the intestinal immune system determines the nature of the antigen-induced innate and adaptive immune response.
Antigen presenting cells; dendritic cells

Although at steady state conditions virtually all antigen-presenting cells may have the capacity to induce antigen-specific T-cells, dendritic cells (DC) appear to be more efficient at this process than others. DC play a crucial role as initiators and modulators of adaptive gastro-intestinal immune responses and probably contain the tolerance “master switch”.

Figure 1: Antigen uptake and recognition by CD4+ T cells in the intestine. Antigens have several strategies by which they can enter the intestine. They might enter through the M-cells in the follicle-associated epithelium (FAE) (a), and after transfer to local DC, might then be presented directly to CD4+ T cells in the PP (b). Alternatively, antigen or antigen-loaded DC from the PP might directly gain access to draining lymph (c), with subsequent T-cell recognition in the mesenteric lymph nodes (MLN) (d). A comparable process of antigen or antigen-presenting cell (APC) dissemination to MLN might occur if antigen enters through the epithelium covering the villus lamina propria (e), but in this case, there is the further possibility that MHC class II+ enterocytes might act as local APC (f). Previously a new route has been described of mucosal dendritic cells which directly sample antigens by intraluminal extensions(g). In all cases, the antigen-responsive CD4+ T cells acquire expression of the α4β7 integrin and the chemokine receptor CCR9, leave the MLN in the efferent lymph (h) and after entering the bloodstream, exit into the mucosa through vessels in the lamina propria. T cells, which have recognized antigen first in the MLN, might also disseminate from the bloodstream throughout the peripheral immune system. Antigen might also gain direct access to the bloodstream from the gut (i) and interact with T cells in peripheral lymphoid tissues (j). Revision of Mowat AM, Nat Rev Immunol. 2003 Apr;3(4):331-41.
Under normal physiological conditions DC are ‘quiescent’ or ‘immature’, capable of presenting antigen and inducing tolerance, but being sufficient responsive to inflammatory stimuli to rapidly mature and allow T cell priming and protective immunity when necessary. The most important feature of DC is their phenotypic and functional plasticity. DC control immune response at the mucosal surfaces by inducing differentiation of naïve CD4+ T cells into either effector or regulatory phenotypes. This polarization depends on the nature of the antigen and the circumstances of DC priming. Activation of DC by microbes is mediated via specific recognition of pathogen associated molecular patterns (PAMP) by pattern recognition receptors (PRR), a set of evolutionary conserved proteins expressed by various cell types including DC. Upon interaction with microbial ligands, pro-inflammatory cytokines or CD40Ligand, DC rapidly acquire an activated phenotype. These mature DC have distinct Th cell polarizing capacities and are able to regulate T cell activation by four distinctive signals: antigen specific peptide presentation via major histocompatibility complex (MHC) class II, cytokine environment, differential expressed costimulatory molecules (B7 family members) and homing receptors (for example CCR9 and α4β7), all contributing to optimal T cell activation. During differentiation DC loose their endocytic capacity, migrate to secondary lymphoid structures and acquire the ability to induce a wide variety of B- and T- cell responses.

In general, several distinct DC subsets are derived from either DC with a myeloid origin (mDC), including various types of interstitial DC, and those with a plasmacytoid origin (pDC), which secrete high amounts of IFN-α upon viral encounters. Other mDC and pDC populations are defined by the expression of cell surface markers such as CD11c, CD8α, CD11b and CD4.

**Tolerogenic dendritic cells**

Dendritic cells play an indispensable role in the induction and maintenance of tolerance. Although the exact phenotypes and functional properties of tolerogenic DC still need to be determined, the tolerogenic function of DC appears to involve various mechanisms including costimulatory molecules, secretion of immunosuppressive cytokines (IL-10 and TGF-β) and an impaired ability to synthesize immunostimulatory cytokines (such as interleukin-12). Although intestinal DC are not inherently tolerogenic, it is believed that, due to the unique local immune environment in the mucosal tissues, DC subsets in the GALT have distinctive immune-modulating capabilities. Recently it has become apparent that several types of tolerogenic DC exist,
including steady state, immature/semimature DC, pathogen-related DC, which can be induced by several micro-organisms and immune-privileged DC, as present in certain anatomical sites as the eye or brain\textsuperscript{39}.

Under steady-state condition, the default pathway of immature DC is the induction of regulatory T-cells. Immature/semimature DC can produce IL-10 and TGF-β, which have been shown to contribute to tolerance induction and the generation of regulatory T cells or anergic T cells\textsuperscript{40, 41}. It should be noted that even in the context of infection, induction of regulatory T cells by DC is thought to be necessary because this limits detrimental tissue damage resulting from the activity of the effector T cells. On the other hand, regulatory T cells will also contribute to immune evasion, promoting the survival and pathogenicity of the invading pathogen. The priming of pathogen related DC for the induction of tolerance could often be ascribed to particular microbial components. For example, tolerogenic DC and subsequent regulatory T cell development has been described by filamentous haemagglutinin A (FHA) of \textit{Bordetella Pertussis}, lysophosphatidylserine (lyso-PS) of \textit{Schistosoma mansoni}, or mannose-capped lipoarabinomannan (ManLAM) of mycobacteria\textsuperscript{42-44}. Moreover several other exogenous signals have been described that are able to induce a tolerogenic DC population, comprising Vitamin D3 metabolite, rapamycine, corticosteroids, cyclosporine A and aspirin\textsuperscript{45}.

Interestingly Treg are also able to directly interact with DC \textit{in vivo}\textsuperscript{46}. After forming aggregates, Treg specifically down-regulate the expression of CD80 and CD86, but not CD40 or class II MHC on DC, leading to a tolerogenic phenotype\textsuperscript{47}. This process is referred to as infectious tolerance, which is believed to allow the expansion of a regulatory environment in a bystander manner. Finally previous work demonstrates that CD3 antibody treatment transiently depletes large numbers of T cells and subsequently induces indirectly long-term immune tolerance\textsuperscript{48}. This seems to be related to enhanced TGF-β production by immature DC and macrophages after engulfment of apoptotic cells, subsequently resulting in induction of Foxp3\textsuperscript{+} Treg\textsuperscript{49, 50}.

Several identified costimulatory and inhibitory pathways comprising an enzyme indoleamine 2,3-dioxygenase (IDO)\textsuperscript{51}, ICOS and PDL1/2\textsuperscript{52, 53}, an integrin CD103\textsuperscript{54, 55} are used by tolerogenic DC, and it is likely that more pathways will be discovered in the short future. Although our understanding concerning these pathways is still rudimentary, it is apparent that a precise balance between all different pathways determines the outcome of T cell responses. These and other recent findings call for a shift in the basic understanding of how the immune system manages tolerance and
indicate that intestinal DC are potential therapeutic targets for induction of oral tolerance or indeed breaking tolerance during oral vaccination.

**Effector and regulatory T cells**

Protective immunity against different classes of pathogens is mediated by different CD4⁺ and CD8⁺ effector T cell types, which accumulate in the gastro-intestinal tract in the lamina propria and within the epithelial cell layer. Their selective homing is dependent on the expression of both the chemokine receptor CCR9 and the integrin receptor α4β7, which binds the mucosal addressin cell-adhesion molecule 1\(^{56-58}\). Many T cell populations in the lamina propria and overlying epithelium display characteristics of an effector type but there is significant heterogeneity with regard to their phenotype and function, for example between γδ T cells and natural killer (NK) T cells. These effector cells are the first to encounter invading pathogens and ensure the GALT to respond rapidly and effectively to repeated assault by enteric pathogens. The CD4⁺ effector T cells are classified in Th1, Th2, Th17 and the development of these effector T cells is orchestrated by DC upon pathogen recognition. This distinction is made because of different functional properties and based on unique cytokine profiles. Effector Th1 cells are characterized by the production of high levels IFN-γ, IL-2, TNF-β and TNF-α. These cytokines are instrumental in cell-mediated immunity against endosomial pathogens such as viruses. Effector Th2 cells are crucial in the clearance of eukaryotic multicellular parasites and characterized by the production of high levels IL-4, IL-5 and IL-13. Recently, a novel subset of effector T cells has been described, Th17, which protects surfaces (e.g., skin, lining of the intestine) against helminths and extracellular bacteria. In mice, IL-6 in combination with TGF-β production induces a Th17, whereas IL-23 serves to expand previously differentiated Th17 cell populations. Moreover, Th17 appear to be important in the pathogenesis of autoimmune diseases\(^{59}\) (Figure 2).

In addition, several T cells with regulatory properties are operational in the gut mucosa, comprising CD4⁺ T cells, CD8⁺ T cells, NK T cells and γδ T cells. These regulatory T cells have the capacity to suppress the proliferation and cytokine production by Th1, Th2 or Th17 cells. Initially CD8⁺ suppressor cells were identified as the regulatory T cell population thought to be involved in oral tolerance\(^{60}\). Different subsets of CD8⁺ cells have been described that may contribute to oral tolerance induction\(^{61-63}\). However their functions have not been clearly defined and there is no absolute requirement for CD8⁺ T cells in the induction or maintenance of
Figure 2: Instruction of immature DC leads to mature DC and consequently T-cell polarization.

The T-cell response generated is fully dependent on the phenotype of effector DC. DC undergo a very flexible program of maturation upon activation by pathogenic motifs and/or environmental signals. Type 1 immunogenic factors present in the mucosal tissue activate immature DC (iDC) to become mature DC (mDC) with the capacity to instruct naïve CD4+ T-cells to become T-helper 1 cells (Th-1), i.e. the expression of ICAM-1 and delta-4 and the secretion of IL-12 and IFN-α/β. Tolerogenic factors will generate tolerogenic dendritic cells (tDC) which are of therapeutic interest in several inflammatory, auto-immune and allergic diseases.

oral tolerance\textsuperscript{64}. Furthermore liver derived NK T cells have been reported to transfer oral tolerance induced by antigen feeding\textsuperscript{65}, suggesting an important immunoregulatory function in oral tolerance for NK T cells. However, oral tolerance can be induced in mice lacking NK T cells\textsuperscript{66}. In some models γδ T cells seem to play a role in oral tolerance. For example low dose oral tolerance can be prevented or even abrogated by depleting γδ T cells \textit{in vivo} and can be transferred by γδ T cells isolated from fed mice\textsuperscript{67, 68}. Moreover, they are thought to play an important homeostatic role in regulating potentially harmful immune responses in the intestine\textsuperscript{69}. These data indicate that mucosal immune activation is regulated at various levels by different cells that downregulate immune responses. A rapidly expanding body of evidence indicates that the most important
among these regulatory cells reside within the CD4\(^+\) T cell population which play an indispensable role in the induction and maintenance of oral tolerance\(^{70, 71}\).

**Regulatory T cells**

Regulatory T cells (Treg) represent a heterogenous population of lymphocytes with the ability to suppress both adaptive and innate immune responses. This characteristic makes them important for both maintenance of immunological tolerance and control of anti-microbial responses\(^{72-74}\). Regulatory T cells downregulate effector cells, including CD4\(^+\) and CD8\(^+\) T cells, natural killer cells, and dendritic cells, at various levels, such as their activation, differentiation, expansion, and even effector function.

Several phenotypically and functionally distinct Treg subsets have been implicated in suppression of intestinal inflammation and induction of oral tolerance. Based on their origin, expression marker profile and cytokine production CD4\(^+\) Treg are divided into three major groups, the so-called thymus-derived ‘naturally occurring’ regulatory T-cells (nTreg), which maintain tolerance to self-antigen under normal physiological conditions and probably play a central role in regulating gut immune homeostasis\(^1, 75, 76\), secondly ‘adaptive’ regulatory T-cells (aTreg), containing the so-called Tr1 and Th3 cells, characterized by the secretion of the anti-inflammatory cytokines IL-10 (Tr1 cells) and/or TGF-β (Th3 cells) after antigen-specific triggering\(^{77-79}\). A distinct category of Treg that acquires Foxp3 upon TGF-β stimulation and are Foxp3\(^+\)CD4\(^+\)CD25\(^{low}\) has been recently identified. These so-called inducible Treg (iTreg) have regulatory functions both in vitro and in vivo\(^{80, 81}\), and represent a different cell lineage from thymic-derived CD25\(^+\) Treg in the periphery but share most of their phenotypical and functional properties and may play an important role in their maintenance\(^{81-83}\). Currently definitive markers of endogenous and converted Foxp3 Treg still lack, making it impossible to distinguish between naturally and inducible Treg (Figure 3).

Treg use a toolbox that contains inhibitory cytokines, like IL-10, TGF-β and the newly discovered IL-35, and can induce suppression by cytolysis, mediated by the excretion of granzymes. Another way to cause suppression is metabolic disruption. Abundant expression of the CD25 Treg may bind all IL-2 leading to starvation of dividing effector T-cells. Finally suppression may involve targeting maturation and/or function of dendritic cells, leading to decreased subsequent effector T-cell instruction\(^{84}\).

It is clear that regulatory T cells are key players of immune regulation, and that they have important functions in suppressing unwanted
inflammatory responses towards self-antigens and the antigens of endogenous intestinal bacteria. Therefore induction of regulatory T cells is a potentially extremely potent therapeutic tool.

**Induction of oral tolerance as therapeutic application**

The induction of antigen specific oral tolerance is an attractive therapeutic objective, because it generally lacks toxicity, can be easily administered over time, and avoids side-effects associated with generalized immune suppressive intervention or avoidance of the causative antigen\textsuperscript{20, 85}. Previously it has been demonstrated that oral administration of (auto) antigens or allergens has been found effective in preventing the induction of autoimmune and allergic diseases in animal models; these diseases include multiple sclerosis (MS), arthritis, uveitis, diabetes in non-obese diabetic (NOD) mice, encephalitis and nickel allergy\textsuperscript{86, 87}. Unfortunately several previous clinical attempts to induce oral tolerance for therapeutic purposes in humans have failed\textsuperscript{88}. These failures may be related to
several factors including the source, the purity, and the amount of (auto)antigen needed and the presentation of the antigen to the mucosal immune system. A major target of immunotherapy for autoimmune and inflammatory diseases is both the induction of Treg that mediate immunological tolerance or the promotion of the inherent tolerogenic DC. Current strategies for therapeutic induction of antigen-specific suppressor cells face significant hurdles, and usually require techniques to isolate, handle and transfer adequate numbers of regulatory cells.

**Lactococcus lactis (Figure 4)**

In this thesis we explore a novel therapeutic approach for the induction of mucosal tolerance by active delivery of recombinant autoantigens or allergens at the intestinal mucosa by genetically modified *Lactococcus lactis* (*L. lactis*). This approach obviates the need for large-scale purification of human (auto)antigens or allergens and thereby circumvents some of the current problems associated with induction of oral tolerance in humans.

*L. lactis* is a non-pathogenic, non-invasive, non-colonizing gram-positive bacterium which has been used for the fermentation of milk products. The food-grade bacteria are Generally Regarded As Safe (GRAS) according to the US Food and Drug Administration. We have produced genetically modified *L. lactis* strains for local synthesis and delivery of immunomodulatory proteins to the intestinal mucosa. This potential

![Figure 4: Lactococcus lactis: gram-positive cocci](image)
use of this bacterium as a treatment for several inflammatory, allergic or autoimmune diseases has many advantages compared to systemic treatment, such as a lower toxicity and a higher biological availability of the preferred compound. Moreover we have established an adequate biological containment system for its clinical application\textsuperscript{94}. A phase I, open label clinical trial with biologically contained \textit{L. lactis} strain secreting human IL-10 was performed in Crohn’s disease patients. 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\textsuperscript{95}. Subsequently we developed a \textit{L. lactis} mediated delivering of low dose mucosal antigen for the therapeutic induction of antigen-specific oral tolerance and evaluated the effect on local and systemic immune responses in different mouse models, comprising wildtype Balb/c, OVA-TCR transgenic (DO11.10) and NOD AB\textsuperscript{O} DQ8 transgenic mice\textsuperscript{96}.

\textbf{Celiac disease}

The chronic, small intestinal inflammation that defines celiac disease is caused by a loss of tolerance to ingested gluten peptides, strongly associated with a HLA-DQ2 or HLA-DQ8 restricted T-cell response\textsuperscript{97}. Disease pathogenesis involves interactions among environmental, genetic, and immunological factors\textsuperscript{98}. The exact pathological mechanism that leads to celiac disease is not known, but it is common knowledge that it develops in genetically susceptible individuals by the dietary ingestion of proline- and glutamine-rich proteins that are found in wheat, rye, and barley and are widely termed “gluten”. To date, celiac disease can only be treated by a socially restrictive diet that requires lifelong abstinence from foods that contain wheat, rye, or barley. While a strict gluten free diet can lead to healing of the intestine the intolerance to gluten is permanent. Although approximately 1-2% of the Caucasian population is affected by celiac disease, only 10\%–15\% or fewer of these individuals have been diagnosed. The symptoms of celiac disease (CD) vary so widely among patients that there is no such thing as a “typical celiac”. In some cases, the disease is relatively asymptomatic, first being detected by antibody screening (for example, of a family member of an affected patient). In other cases a spectrum of intestinal and/or extraintestinal symptoms can occur, like diarrhoea, recurring abdominal bloating and pain, anemia, fatigue and/or depression. The amount of intestinal damage that has occurred and the length in time of abnormal nutrient absorption seem to be the factors that determine the type and severity of symptoms experienced. Life-threatening complications, although relatively rare, can
include the development of refractory celiac disease and enteropathy-associated T cell lymphomas (EATLs).

Celiac disease may be an especially attractive target for the induction of antigen-specific oral tolerance as a therapeutic objective due to extensive immunological knowledge about the disease and the ability of the L.lactis to deliver the antigen at the site of the primary response to achieve both direct and bystander tolerance. Tolerance to multiple immunodominant epitopes may be induced by using multiple L.lactis each secreting different immunogenic peptides or engineering a bacterium that secretes several peptides.

**Inflammatory bowel disease (IBD)**

The intestinal immune system is in a constant state of controlled inflammation, and there is substantial evidence that a loss of control is an important pathogenic mechanism in inflammatory bowel diseases (IBD). A major current working hypothesis defines Crohn’s disease as a dysregulated immune response towards components of the intestinal flora, leading to chronic intestinal inflammation. The causes for this inappropriate response can be attributed to (a combination of) genetic predisposition, defects in the epithelial barrier, the innate immune response or the adaptive immune response. Unfortunately it is still unknown what antigens are involved in the pathogenesis of IBD. Therefore, in order to be able to suppress immune responses as a therapeutic application, antigen non-specific Treg should be induced functioning through the so-called bystander suppression induced by generation of “bystander” regulatory T cells. It is now clear that regulatory T cells do not need to be antigen specific in order to suppress immune responses as a result of so-called bystander suppression. A clear example of bystander suppression was demonstrated in the SCID (severe combined immunodeficient) transfer model, where OVA-specific Tr1 cells did suppress the occurrence of IBD after administration of OVA, although OVA is not involved in the immune mediated inflammation in this model. Therefore, the OVA-specific Tr1 cells were able to suppress responses induced by other antigens, very likely derived from intestinal bacteria, and this is known as “bystander” suppression. In various situations CD4+CD25+ regulatory T cells, once activated by their TCR, have been shown to be capable of such antigen-non-specific bystander suppression.
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**Scope and outline of this thesis**

The studies described in this thesis have been performed to develop therapies for several autoimmune, inflammatory and allergic diseases that result from a pathogenic antigen-driven immune response. Active delivery of IL-10, (auto)antigens or allergens by genetically modified *Lactococcus lactis* (*L. lactis*) would provide a novel therapeutic tool for the induction of tolerance.

The goal of the experiments in **chapter two** was to investigate whether *Lactococcus lactis* secreting human IL-10 (LL hIL-10) induce tolerance via the modulation of dendritic cell- and subsequent T cell function *in vitro*. Human peripheral blood monocyte-derived DC were incubated with viable *L.lactis* or *L.lactisIL-10* and maturation factors (MF; IL-1β, TNF-α and LPS) and used to stimulate highly purified naïve T cells to assess the nature of adaptive immune responses. T cells generated by mature DCs exposed to *L.lactisIL-10* and MF showed the profound ability to suppress the proliferation of bystander T cells in an *in vitro* suppressor assay. This suppression was dependent on full maturation of DC, as DC exposed to *L.lactisIL-10* in the absence of MF did not induce suppression. Furthermore, both *L.lactisIL-10*-exposed DC and the regulatory T cells they induced showed enhanced production of IL-10, which was instrumental in the induction, but not the function, of regulatory T cells.

In **chapter three**, we show that active delivery of recombinant antigen at the intestinal mucosa by genetically modified *L. lactis* (LL-OVA) induces suppression of local and systemic OVA specific T cell responses in DO11.10, mediated by induction of CD4+CD25- regulatory T cells that function through a TGF-β dependent mechanism. Our data indicate that the mode of mucosal delivery of an antigen critically determines immune activation, as orally administered OVA did not induce tolerance. This approach may be used for the development of effective and non-toxic treatment of several autoimmunity and allergic diseases.

In **chapter four**, we hypothesized that downregulation of regulatory T cell (Treg) function, by TGF-β1 neutralisation, interferes with induction of oral tolerance, and hence could enhance vaccine immunogenicity. We therefore studied the effect of P17, a short peptide that inhibits TGF-β1, on Treg activity *in vitro* and *in vivo*. *In vitro* studies showed that P17 inhibited murine and human Treg-induced unresponsiveness of effector T cells. Administration of P17 to mice immunized with peptide vaccines containing tumor or viral antigens enhanced anti-vaccine immune
responses, improving protective immunity against tumor growth or viral infection/replication. Moreover, P17 prevented development of immune tolerance induced by mucosal delivery of an antigen, by LL-OVA in DO11.10 mice. Thus, inhibition of TGF-β with a short synthetic peptide potentiates immune responses, an effect that can be exploited to enhance vaccination efficacy.

In chapter five, we tested the efficacy of the genetically modified L. lactis secreting a gliadin derived deamidated DQ8 epitope which is immunodominant in celiac disease and demonstrated that its mucosal delivery by genetically modified L. lactis, induces suppression of local and systemic DQ8 restricted T-cell responses in NOD ABo DQ8 class II transgenic mice, a well established genotypic celiac disease mouse model. 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-β and associated with a significant induction of Foxp3+ regulatory T-cells. Because this approach of antigen-charged probiotics has the capacity for potentiating oral tolerance even in the setting of established hypersensitivity, it may be applicable for the treatment of celiac disease and possibly other autoimmune and/or allergic diseases.

In chapter six, we demonstrate differences in immunogenicity of L. lactis between Balb/c and BL/6 mice. Because of the occurrence of an 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.

In chapter seven, we discuss Treg in IBD and their exploitation in therapy. In mice, a loss of Treg activity results in inflammatory bowel disease and their therapeutic application in various murine models shows promising results. In human inflammatory bowel disease, Treg activity has not been thoroughly studied, but currently available data do not provide evidence for a loss of Treg activity, but apparently, the regulatory capacity of these cells is insufficient to down-regulate inflammation. This review discusses evidence for abnormal regulation of T cell activation in Crohn’s disease, as well as data pertaining to the existence and functional activity of
regulatory T cells in the intestinal mucosa. Furthermore, we consider the potential therapeutic application of regulatory T cells in IBD.

In chapter eight publicity highlights are given comprising the editorial in Gastroenterology and some articles in several different newspapers.

Finally chapter nine and ten give a closing summary and discussion of this thesis.

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Chapter 1


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