Antigen-specific oral tolerance for the treatment of inflammatory and allergic diseases
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We developed a novel effective experimental therapy for inflammatory diseases, by using, genetically modified bacteria, *Lactococcus lactis* that synthesize and secrete very low doses of antigen (protein) at the site of the intestinal mucosa thereby inducing antigen-specific tolerance. This approach has been shown to be very effective in the preclinical setting and offers a great opportunity for further development as a therapy for mucosal and systemic autoimmune, inflammatory and/or allergic diseases.
Antigen-specific oral tolerance for the treatment of inflammatory and allergic diseases

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

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Ter nagedachtenis aan mijn Oma, dokter H.G. Meijerink,
die ik nog dagelijks mis.
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List of abbreviations

AICD: Activation induced cell death
APC: Antigen presenting cell
CD: Crohn’s disease
CFA: Complete Freund’s Adjuvant
CFSE: 5,6-carboxy-succinimidyl-fluoresceine-ester
CFU: Colony forming units
CLN: Cervical lymph node
CTLA-4: Cytotoxic T-lymphocyte associated antigen 4
DC: Dendritic cell
  bmDC: Bone marrow derived dendritic cell
  mDC: Myeloid dendritic cell
  pDC: Plasmacytoid dendritic cell
DTH: Delayed type hypersensitivity
EC: Epithelial cells
FHA: Filamentous Hemagglutinin A
Foxp3/FOXp3: Forkhead box P3
γδ T cells: Gamma-delta T cells
GALT: Gut-associated lymph node tissue
GITR: Glucocorticoid-induced TNFR family-related protein
IBD: Inflammatory bowel diseases
IDO: Indoleamine 2,3-dioxygenase
IEL: Intra-epithelial lymphocyte
IFN-γ: Interferon-gamma
i.g.: Intra-gastric
IL: Interleukin
IPEX: Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked
LAP: Latency-associated peptide
L. lactis: Lactococcus lactis
LL: Lactococcus lactis
LL-eDQ8d: Genetically modified L. lactis secreting deamidated DQ8 peptides
LL-OVA: Genetically modified L. lactis secreting ovalbumin
LP: Lamina propria
LPS: Lipopolysaccharide
MALT: Mucosal-associated lymphoid tissue
M-cell: Microfold cell
MDP: Muramyl dipeptide
MFI: Mean Fluorescence Intensity
MHC-II: Major histocompatibility complex
MLN: Mesenterial lymph node
MLR: Mixed leucocyte reaction
NK cells: Natural killer cells
NOD: Nucleotide oligomerisation domain
NOD (mice): Nonobese diabetic
OVA: Ovalbumin
PAMP: Pathogen-associated molecular patterns
PBMC: Peripheral blood mononuclear cell
PE: Phycoeythrin
PerCP: Peridinin chlorophyll protein
PGN: Peptidoglycan
PP: Peyer's patches
PRR: Pattern recognition receptor
s.c.: Subcutaneous
SCID: Severe combined immune deficient
SNP: Single nucleotide polymorphism
TCR: T cell receptor
Tg: Transgenic
TGF-β: Transforming growth factor β
Th: T helper cell
TLR: Toll-like receptor
TNF: Tumor necrosis factor
Treg: Regulatory T-cell
nTreg: Naturally occuring regulatory T-cell
aTreg: Adaptive regulatory T-cell
iTreg: Induced regulatory T-cell
UC: Ulcerative colitis
General introduction

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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
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 cells 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 ligand.

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 immunity. The GALT consists of organized lymphoid structures such as Peyer’s Patches (PP) which are large aggregates of lymphoid tissue found in the small intestine, mesenterial lymph nodes (MLN), isolated lymphoid follicles (distributed throughout the wall of the intestines), and isolated immune cells, predominantly lymphocytes, scattered throughout the epithelium and lamina propria. 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 tolerance. 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 secretion. Nonetheless a significant antigen load is capable of entry into the mucosa. A next line of defense 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, etc.).
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 interfollicular 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 naïve 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”.
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,
General introduction

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\(^39\). 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\(^40, 41\). 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 *Bordetella Pertussis*, lysophosphatidylserine (lyso-PS) of *Schistosoma mansoni*, or mannose-capped lipoarabinomannan (ManLAM) of mycobacteria\(^42-44\). 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\(^45\).

Interestingly Treg are also able to directly interact with DC \textit{in vivo}\(^46\). 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\(^47\). 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\(^48\). 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\(^+\) Treg\(^49, 50\).

Several identified costimulatory and inhibitory pathways comprising an enzyme indoleamine 2,3-dioxygenase (IDO)\(^51\), ICOS and PDL1/2\(^52, 53\), an integrin CD103\(^54, 55\) 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. 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 endosomal 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 (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. Different subsets of CD8+ cells have been described that may contribute to oral tolerance induction. However their functions have not been clearly defined and there is no absolute requirement for CD8+ T cells in the induction or maintenance of
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
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among these regulatory cells reside within the CD4+ T cell population which play an indispensable role in the induction and maintenance of oral tolerance70, 71.

**Regulatory T cells**

Regulatory T cells (Treg) represent a heterogeneous 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 responses72-74. 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 homeostasis1, 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 triggering77-79. A distinct category of Treg that acquires Foxp3 upon TGF-β stimulation and are Foxp3+CD4+CD25low has been recently identified. These so-called inducible Treg (iTreg) have regulatory functions both in vitro and in vivo80, 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 maintenance81-83. 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 instruction84.

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. 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. Unfortunately several previous clinical attempts to induce oral tolerance for therapeutic purposes in humans have failed. These failures may be related to
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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\textsuperscript{89}. 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\textsuperscript{90}. 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 hereby 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\textsuperscript{91-93}. This potential
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. A phase I, open label clinical trial with biologically contained L. lactis strain secreting human IL-10 was performed in Crohn’s disease patients. This trial demonstrated that treatment of humans with viable L. lactis secreting IL-10 is clinically and biologically safe and consequently oral administration of genetically modified L. lactis for intestinal delivery of proteins is a clinically feasible strategy. Subsequently we developed a 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 DQ8 transgenic mice.

**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. Disease pathogenesis involves interactions among environmental, genetic, and immunological factors. 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\textsuperscript{97}. Tolerance to multiple immunodominant epitopes may be induced by using multiple \textit{L.lactis} each secreting different immunogenic peptides or engineering a bacterium that secretes several peptides.

\textbf{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\textsuperscript{99}. 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\textsuperscript{100}. 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\textsuperscript{101}. 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 CD$^+$CD25$^+$ regulatory T cells, once activated by their TCR, have been shown to be capable of such antigen-non-specific bystander suppression\textsuperscript{102}.
General introduction

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


Genetically Modified *Lactococcus lactis* for Delivery of Human Interleukin-10 to Dendritic Cells

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Abstract

The effective ability of dendritic cells (DC) to initiate different types of T cell responses upon different maturation conditions makes this cell-type an important target for innovative strategies requiring either polarized immunity (i.e. in vaccination or cancer) or tolerance (i.e. in chronic inflammatory diseases). We describe a novel DC targeted strategy to induce T-cell mediated tolerance by genetically engineered non-pathogenic Lactococcus lactis expressing human IL-10 (L.lactis\textsuperscript{IL-10}). Monocyte-derived DC were incubated with viable L.lactis or L.lactis\textsuperscript{IL-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 generated. T cells generated by mature DC exposed to L.lactis\textsuperscript{IL-10} markedly suppressed the proliferation of bystander T cells. The regulatory T cells generated by L.lactis\textsuperscript{IL-10} were dependent on full maturation of DC, as DC exposed to L.lactis\textsuperscript{IL-10} in the absence of MF did not induce suppression. Furthermore, both L.lactis\textsuperscript{IL-10}-matured DC and regulatory T cells showed enhanced production of IL-10, which was instrumental in the induction, but not the function, of regulatory T cells. These data further indicate that delivery of anti-inflammatory and immune-stimulatory agents by food-grade bacteria may be a promising strategy in the therapy of intestinal mucosal disease.

Introduction

Dendritic cells (DC) are short-lived regulators of the adaptive immune system controlling both peripheral tolerance and immune activation[1]. Immature DC continuously repopulate the mucosal tissue of the gastrointestinal tract and are able to sense luminal microbial antigens via transepithelial dendrites[2]. Upon activation by pathogens and through environmental signals DC undergo a very flexible program of maturation. During this differentiation process, DC lose their endocytic capacity, migrate to secondary lymphoid structures and acquire the ability to induce a wide variety of T cell responses. Active immunity is induced by pathogenic motifs and danger signals in the local environment while in the absence of pro-inflammatory signals or presence of anti-inflammatory signals, like IL-10, DC are tuned for maintaining peripheral tolerance[3,4 and 5].

Because DC instruct many types of lymphocytes they are an important target for several approaches of immuno-therapy including long-term vaccination, graft-versus-host disease, cancer and autoimmune disease[6].
Several strategies have been designed to modulate DC function in order to induce regulatory T cell responses, including DEC-205, Vitamin D3, Lyso-PS, FHA and interleukin-10 (IL-10) [7-11]. The disadvantage of these immunomodulatory agents is that efficacy may be lost in vivo. [12-14]. We have engineered a non-pathogenic bacterium, Lactococcus lactis, to secrete human IL-10 (L.lactisIL-10), this approach is a realistic and safe therapeutic option in humans [15]. We have examined the immunoregulatory properties of L.lactisIL-10 by coculturing human monocyte-derived DC with viable L.lactisIL-10. We show that L.lactisIL-10 matured DC but not L.lactis matured DC produce enhanced levels of IL-10 and prime naïve T cells to become IL-10 producing suppressor T cells.

Results

L.Lactis IL-10 primes DC to promote the development of regulatory T cells

IL-10 is well known for its immunoregulatory effects on DC resulting in low expression of costimulatory molecules and, as a consequence, low T cell stimulatory capacity. Therefore, we investigated to what extend L.lactisIL-10 affects DC function and primes DC to promote the development of regulatory T cells from naïve precursors. To this aim, immature DC were activated in the presence of L.lactisIL-10, L.lactis and/or IL-10 and subsequently co-cultured with naïve T cells to induce the differentiation of effector T cells. These effector T cells were analyzed for their regulatory potency in a T cell suppressor assay. This assay utilizes two cell-cycle tracking dyes to distinguish between DC-primed T cells (effector T cells) and CD4+ peripheral T cells (responder T cells)[16]. The mean fluorescence intensity of responder T cells during culture with effector T cells derived from MF (MF; TNF-α, IL-1β and LPS) matured DC was taken as 100%. Compared to this reference, only 33% of all responder T cells went into cell cycle progression when L.lactisIL-10 and MF were present during DC maturation (Figure 1a). When immature DC were activated with L.lactis and MF 67% of all responder T cells went into cell cycle progression (Figure 1a). The mean inhibition from five separate experiments was 42% and 72% for L.lactisIL-10 and L. lactis respectively (Figure 1b).

The question remains whether the spatially restricted production of IL-10 is crucial for the induction of regulatory DC or that other factors resulting from genetic manipulation might play a role. Therefore, DC were activated
with *L. lactis* in the presence of increasing doses of recombinant human IL-10.

Compared to the reference situation of MF matured DC (taken as 100%), effector T cells from *L. lactis* matured DC were able to reduce the proliferation of responder T cells from 100% to 78%. However, increasing doses of recombinant human IL-10 (0.5 ng/ml, 5 ng/ml and 50 ng/ml) increased the suppressor capacity of DC-instructed effector T cells to 67.5%, 46.5% and 48.5% respectively (Figure 1c).

Thus, *L. lactis* is able to induce the differentiation of regulatory DC with subsequent generation of T cells that have the capacity to suppress the proliferation of bystander (responder) T cells. The ability of *L. lactis* to induce regulatory immune responses is amplified by the presence of IL-10 during DC activation.
**L. lactis**IL-10 induces the generation of IL-10-producing DC and IL-10-producing T cells

We determined the number of bacteria needed for optimal IL-10 induction in DC in our system. To this aim, *L. lactis*IL-10 was grown overnight at 37°C, diluted 1:50 and grown for three hours at exponential growth phase (approximately 1x10^7 cfu/ml). Subsequently, serial dilutions were made and bacteria were grown for four more hours in the absence of DC (Figure 2a). A dilution of 10^5 cfu/ml of *L. lactis*IL-10 produced the highest amount of IL-10 (81 pg/ml). At higher concentrations of bacteria lower concentrations of IL-10 were detected, most likely due to acidification of the supernatant and instability of IL-10 at lower pH.

To elucidate the role of bacterial IL-10, we analyzed the production of IL-10 during the incubation of immature DC and bacteria and after restimulation of mature DC and DC-instructed T cells. Culture supernatants from immature DC were harvested at 4 and 48 hours after activation of the DC with *L. lactis* or *L. lactis*IL-10 to determine the amount of bacterial IL-10 (at 4 hours) and total IL-10 (at 48 hours) produced. Only 130 pg/ml IL-10 was detected during the first four hours of culture with viable *L. lactis*IL-10 while no IL-10 was found in the cultures with *L. lactis* (Figure 2b). Given the short time course, the IL-10 detected must have been secreted by *L. lactis*IL-10. After 48 hours, total IL-10 production was 1200 pg/ml in DC-cultures containing *L. lactis*IL-10 compared to 680 pg/ml in DC-cultures containing *L. lactis* (Figure 2b).

To assess the ability of DC to produce IL-10 upon encounter with T cells we restimulated DC after activation of DC with *L. lactis* or *L. lactis*IL-10. DC activated by *L. lactis*IL-10 produced 3.2 ng/ml IL-10 upon restimulation with CD40L (Figure 2c), but DC activated by maturation factors only produced 0.1 ng/ml IL-10 and DC activated by *L. lactis* produced intermediate amounts of IL-10 (Figure 2c).

The production of IL-12p70 was comparable between various activated DC, ranging from 0.26 – 0.75 ng/ml, and these amounts are significantly lower compared to the production of IL-12p70 by immature DC (3.6 ng/ml) (Figure 2c).

To analyze the cytokine production of *L. lactis*IL-10 activated DC-instructed T-cells, we isolated the T cells from the mixed lymphocyte culture and subsequently stimulated them with CD3 and CD28. Effector T cells primed by *L. lactis*IL-10 activated DC produced 2.4 ng/ml IL-10 while effector T cells co-cultured with *L. lactis* activated DC produced 0.7 ng/ml IL-10 (Figure 2d). The IL-10 production by *L. lactis*IL-10 generated T cells could be reversed by the addition of neutralizing IL-10 antibodies during the activation of DC with *L. lactis*IL-10 (Figure 2d). Moreover, T cells instructed
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**Figure 2.** *L. lactis*<sup>IL-10</sup> induces the generation of IL-10 producing DC and IL-10 producing T cells. a. *L. lactis*IL-10 was grown overnight at 37°C, diluted 1:50 and grown for three hours at exponential growth phase (approximately 1x107 cfu/ml). Subsequently, a serial dilution was made and bacteria were grown for four more hours in the absence of DC. Subsequently IL-10 was measured in supernatant, mean values with standard error of mean are given. b. Immature DC were activated with 1x105 CFU per ml of viable *L. lactis*<sup>IL-10</sup>, *L. lactis*, *L. lactis*<sup>IL-10/α-IL-10</sup> or *L. lactis*<sup>IL-10/rat IgG1</sup> in the presence of MF. Supernatants were taken after 4 (black bars) and 48 (white bars) hours to assess bacterial IL-10 and total IL-10 production respectively. Mean values with standard error of mean are given; nd indicates not detected; * indicates p<0.05 and ** indicates p<0.01 using heteroscedastic Student t-test. c. Immature DC were left unstimulated, activated with MF or activated with 1x105 cfu of viable *L. lactis*IL-10, *L. lactis*, *L. lactis*<sup>IL-10/α-IL-10</sup> or *L. lactis*<sup>IL-10/rat IgG1</sup> in the presence of MF. DC were harvested after 48 hours activation, washed and restimulated for 48 hours with CD40L transfected irradiated hybridoma cells to assess IL-10 production or in combination with IFN-γ to assess IL-12p70 production. Mean values with standard error of mean are given; * indicates p<0.05 and ** indicates p<0.01 using heteroscedastic Student t-test. d. Immature DC were left unstimulated, activated with MF only or activated with 1x105 CFU of viable *L. lactis*<sup>IL-10</sup>, *L. lactis*, *L. lactis*<sup>IL-10/α-IL-10</sup> or *L. lactis*<sup>IL-10/rat IgG1</sup> in the presence of MF. Mature DC were subsequently cultured with naïve T-cells, these DC-derived T-cells were washed and restimulated with CD3/CD28. Subsequently IL-10 and IFN-γ production was measured. Mean values with standard error of mean are given; ** indicates p<0.01 using heteroscedastic Student t-test.

by *L. lactis*<sup>IL-10</sup>- or *L. lactis*-primed DC also produced high amounts of IFN-γ (4.2 and 4.3 ng/ml respectively) (Figure 2d).

In conclusion, very low levels of spatially restricted bacterial IL-10 during initial activation of immature DC are sufficient to amplify IL-10 by mature DC and effector T cells.

**The induction of regulatory DC by *L. lactis*<sup>IL-10</sup> is dependent on full maturation of DC**

Maturation of DC is important for migration to regional lymph nodes, MHC-presentation, expression of co-stimulatory molecules and the induction of effector T cell responses. DC matured with *L. lactis*<sup>IL-10</sup> in the absence of MF appeared to lack regulatory capacities as 99% of all responder T cells went into cell cycle progression (Figure 3a). As shown
Cell-based delivery of IL-10

Figure 3. The induction of regulatory DC by *L. lactis*IL-10 is dependent upon full maturation of DC. a. Immature DC were matured with MF or 1x10^5 CFU of viable *L. lactis*IL-10 with and without MF. After 48 hours DC were subsequently cultured with naive T-cells, these DC-derived T-cells (test cells) were washed and labeled with PKH. Test cells were stimulated with CD3/CD28 (1:5000/1:2000) and after 16 hours CSFE labeled CD4+ T cells (responder cells) were added (T cell suppressor assay). After 5 days T cells were harvested and cell cycle progression analyzed by flow cytometry. Depicted is the cell cycle progression of responder cells stimulated in the presence of MF derived test cells (white histograms) and of responder cells stimulated by *L. lactis*IL-10 or *L. lactis*IL-10/MF derived test cells (gray histograms). b. Immature DC were left unstimulated (gray histogram), stimulated with *L. lactis*IL-10 (continuous line) or with *L. lactis*IL-10/MF (discontinuous line). Maturation status after 48 hours was assessed by CD83 and CD86 expression.

earlier the presence of MF and *L. lactis*IL-10 during DC maturation induced effective regulatory T cells as only 39% of all responder cells went into cell cycle progression (Figure 3a). Subsequently, maturation status of DC matured by *L. lactis*IL-10 in the presence and absence of MF was compared (Figure 3b). Regulatory DC activated by *L. lactis*IL-10 and MF were CD83^+^ and CD86^{high}, while DC activated by *L. lactis*IL-10 alone were CD83^-^ and CD86^{low} (Figure 3b).

Apparently, full maturation of DC activated by *L. lactis*IL-10 is needed for the induction of regulatory DC and regulatory immune responses.

**IL-10 production by regulatory DC is essential for the induction of regulatory immune responses**

To delineate the role of IL-10 in the generation of regulatory immune responses blocking experiments with an antibody directed against IL-10
were performed. As discussed earlier, bacterial IL-10 was important for the induction of effective regulatory DC (Figure 4a) and addition of αIL-10 during initial activation of immature DC reduced the suppressor activity (Figure 4a). When αIL-10 was added to the mixed culture of \textit{L. lactis} \textsuperscript{IL-10} induced regulatory DC and naïve T cells the inhibition was lost and all (100%) responder T cells went into cell cycle progression (Figure 4a). However, αIL-10 had no inhibitory effect on regulatory T cells during the culture of \textit{L. lactis} \textsuperscript{IL-10} induced regulatory T cells and responder T cells as only 52% of all responder T cells went into cell cycle progression (Figure 4a). In addition, neither αTGF-β nor αCTLA-4 were able to abrogate the regulatory activity of \textit{L. lactis} \textsuperscript{IL-10} induced effector T cells during the suppressor-assay (Figure 4b). On the contrary when effector T cells and responder T cells were cultured separately in a transwell system the suppressor function of effector T cells was complete abrogated (Figure 4b).

These results suggest that IL-10 production by \textit{L. lactis} \textsuperscript{IL-10}–primed regulatory DC is essential for the induction of suppressor T-cells while IL-10 production by suppressor T-cells was not required for the suppressive effect.

\section*{Discussion}

IL-10 is a well-known immunomodulator that is able to reduce the maturation and cytokine production of immature DC\cite{17}. Treatment of immature DC with IL-10 results in the induction of antigen-specific anergic T cells\cite{18, 19}. These T cells appear to be able to suppress the activation and proliferation of allogenic T cells in a contact dependent, antigen specific manner that is mainly dependent on CTLA-4 expression but not on IL-10 production by T cells\cite{20}.

In the present study we demonstrate that activation of DC with the combination of spatially restricted IL-10 and a non-pathogenic microorganism primes regulatory DC. \textit{L. lactis} \textsuperscript{IL-10} induced regulatory DC, provided they are fully matured, produce high amounts of IL-10 and promote the development of regulatory T cells from naïve precursors. A common function of many different types of regulatory T cells described is the ability to suppress the proliferation and cytokine production of bystander T cells. Here we describe yet another type of regulatory T cells which, in some aspects, is different from those earlier described. These regulatory T cells instructed by \textit{L. lactis} \textsuperscript{IL-10}–exposed DC are able to induce suppression in the absence of DC and/or bacteria and produce large amounts of both IL-10 and IFN-γ. In addition, their effects are not mediated via TGF-β or CTLA-4. Because \textit{L. lactis} \textsuperscript{IL-10} derived effector T
cells lost their suppressive capacity when cultured in a transwell system.

It is likely that yet undefined contact-dependent factors mediate their suppressive effect on CD4+ T cells. Apparently, IL-10 production by regulatory DC is essential for the induction of regulatory T cells while IL-10 production of regulatory T cells was not needed for the suppressive effect. The exact function of IL-10 production by regulatory T cells is not known but might be important for the persistence of regulatory immune responses through feed-back on newly recruited immature DC in the periphery in vivo[21].
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*L. lactis* is a food-grade gram-positive bacterium that is used for food fermentation and it is regarded as intrinsically safe for human use. The utilization of *L. lactis* for delivery of proteins [22, 23] has several advantages including the fact that like other lactic acid bacteria, *L. lactis* has intrinsic characteristics able to suppress immune responses via modulation of DC[24]. In our experiments we found that the development of regulatory T cells could be prevented by the addition of anti-IL-10 antibodies during co-culture of *L. lactis*-*IL-10*/immature DC and mature DC/naive T cells. This suggests a possible IL-10 mediated induction of suppressor immune responses by *L. lactis*-*IL-10*.

Lactic acid bacteria are able to temporarily divide and reside within the gastrointestinal tract maintaining a continuous production of specific proteins. Thus, we propose a novel cell-based strategy to target DC function; bacteria are employed to spatially restrict the delivery of recombinant proteins at the cell surface of target cells, in this case DC. Treatment of human beings with recombinant bacteria is a novel and highly promising avenue to treat immune mediated gastro-intestinal disease. A phase I study in patients with Crohn’s disease has shown that genetically engineered bacteria are clinically safe and biologically contained[25].

In conclusion, we provide evidence for a cellular mechanism as to how genetic engineered *L. lactis* may be adapted for the induction of regulatory immune responses in humans. Spatially restricted production of IL-10 by genetically engineered *L. lactis* modulates DC function in a regulatory manner. The observation that genetic modified bacteria are able to deliver biologically active proteins to human cells opens several possibilities to develop other therapeutic strategies. It might for example be interesting to test biologically active proteins not only for immuno-regulation in chronic inflammation but also for growth-regulation in cancer.

**Materials and methods**

**Bacterial strains**

For the generation of *Lactococcus lactis* MG1363 IL-10 (*L. lactis*-*IL-10*) and *Lactococcus lactis* MG1363 (*L. lactis*) the plasmid pOTHY12 was used. For *L. lactis*-*IL-10*, a 1 Kb region including at its 3’ end the constitutive thyA promoter (PthyA) from *L. lactis* MG1363 precedes a fusion gene between the usp45 secretion leader and hIL-10. This leads to a functional
Cell-based delivery of IL-10

coupling of PthyA to usp45-hIL-10, expression of the precursor, correct N-terminal processing of the precursor and secretion of mature hIL-10. In optimised in vitro growth, L. lactis MG1363 pOTHY12 will produce in its culture supernatant approximately 1 µg hIL-10 per 2x10^9 bacteria. In pOTHY12, the hIL-10 gene is followed by a region identical to the 1Kb segment downstream of the thyA gene. Both L. lactisIL-10 and L. lactis were grown overnight at 37°C (Elbanton incubator) in M17 broth (Difco) supplemented with 0.5% glucose and 50 µg/ml erythromycin (Abbott, Saint-Rémy-sur-Avre, France). Bacteria were diluted 1:50 in IMDM 5% FCS and grown for three hours at 37°C and harvested at exponential growth phase (approximately 1x10^7 cfu/ml).

**Generation and maturation of DC**

All cultures were performed in Iscove’s modified Dulbecco’s medium (IMDM) with 1% FCS (HyClone, Lagan, UT) and erythromycin (50 µg/ml, Abbott). Peripheral blood of healthy volunteers was used to generate immature DC; monocytes were isolated after Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) and Percoll (Amersham Biosciences AB, Uppsala, Sweden) gradient centrifugation. Then, 5x10^5 monocytes were cultured in the presence of recombinant human (rhu) GM-CSF (500 U/ml; a gift from Schering-Plough, Uden, The Netherlands) and rhuIL-4 (250 U/ml; Pharma Biotechnology). After 6 days, cultures consisted of CD14^−, HLA-DR^+, CD83^−, CD86^low and CD40^high immature DC. These cells produce high amounts of IL-12p70 upon stimulation with CD40L and are called immature DC. Maturation was achieved at day 6 by adding 1x10^5 cfu of the above-mentioned bacteria with or without the following stimuli: recombinant human (rhu) IL-1β (5 ng/ml; Boehringer Mannheim, Germany), rhu TNF-α (25 ng/ml; PBH, Hannover, Germany), LPS (Sigma) and rhu IL-10 (Schering-Plough, Uden, The Netherlands). After 4 hours of DC culture with viable bacteria, 50 µl of supernatant was harvested for measurement of bacterial IL-10 (direct cytokine measurement; CLB, Amsterdam, The Netherlands) and gentamycin (86 µg/ml, Sigma) was added to prevent bacterial overgrowth. On day eight (mature) DC were harvested and washed twice, culture of DC on M17 agars confirmed that bacterial killing was complete. Mature DC were stimulated (2 x 10^4 cells in 200 µl) with one of the following stimuli: IFN-γ (gift from Dr. P van der Meide; U-Cy tech, Utrecht, The Netherlands) and/or CD40L-transfected J558 plasmacytoma cells (gift from Dr. P. Lane, Birmingham Medical School, Birmingham, U.K.). After 48 hours stimulation, supernatants were used for cytokine detection using ELISAs for IL12p70 (R&D Systems, detection limit 31.2 pg/ml) and IL-10 (CLB, Amsterdam, The Netherlands).
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**T cell priming by mature DC**

Mature DC (5x10³ cells/200 µl) were incubated with 2.5 x 10⁴ highly purified CD4⁺CD45RA⁺CD45RO⁻ naïve T cells (>90% as assessed by flow cytometry) from PBMCs using MACS separation system (Miltenyi Biotech, Germany). When indicated rat-anti-human IL-10 neutralizing IgG₁ (1:1000, BD Pharmingen, San Jose, CA, USA) antibodies were added to the mixed lymphocyte reaction, purified rat IgG₁ (BD Pharmingen) was used as isotype control. After adequate priming, T cells were further expanded in IMDM 10% FCS containing IL-2 (10 U/ml, Cetus, Emeryville, CA) and IL-15 (10 ng/ml, R&D Systems). When proliferation was halted, T cells were harvested and used for restimulation with CD3 and CD28 or suppressor T cell assay.

**T cell restimulation and cytokine measurement**

T cells (100.000 cell/200 µl) were stimulated with soluble mouse anti human-CD3 and mouse anti human-CD28 (both CLB, Amsterdam, The Netherlands), final dilution 1:1000. After 48 hours stimulation, supernatants were used for cytokine detection using ELISAs for IFN-γ (R&D Systems, detection limit 31.2 pg/ml) and IL-10 (CLB).

**T cell suppressor assay**

Non-proliferating DC primed T cells (test cells) were harvested and washed three times with serum-free IMDM. Test cells (5x10⁵) were stained with 3x10⁻⁵ M PKH-26 (Sigma), a red cell cycle tracking dye, for 5 minutes at room temperature according to the manufacturer’s instructions. After thorough washing, 2.5x10⁴ T cells were pre-activated overnight with anti-CD3 (1:5000) (CLB, Amsterdam, The Netherlands) and anti-CD28 (0.5 µg/ml) in round-bottom 96-well plates. For transwell experiments, 2x10⁶ PKH labelled test cells were put in the upper compartment of a 24-well plate transwell system (Corning Inc.). The following day CD4⁺ T cells (responder cells) were labeled with CFSE (0.5 mM, Molecular Probes Inc., Eugene, OR), a green cell cycle tracking dye, for 15 minutes at room temperature. After 5 days the cellular content of CFSE in the responder cells was analyzed by flowcytometry. As indicated, rat-anti-human IL-10 (BD Pharmingen), goat-anti-human TGF-β (R&D, Minneapolis, USA) and mouse-anti-human CTLA-4 (Bioscience) were used as neutralizing antibodies.
**Statistics**

For comparison of cytokine production a heteroscedastic Student t-test was performed, when multiple groups were present a Kruskall-Wallis One Way Analysis of Variance was performed (SPSS, version 11.01, Chicago, IL, USA). Statistical significance was defined as a p<0.05, confidence interval 95%.

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**References**

Chapter 2


Induction of OVA-specific tolerance by oral administration of Lactococcus lactis secreting OVA

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Abstract

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

Introduction

The mucosal immune system maintains an equilibrium between tolerance towards commensals and harmless agents (e.g. food antigens) on the one hand and active immunity towards pathogenic agents on the other. Disturbance of this balance is an important pathogenic mechanism in many different autoimmune, allergic and inflammatory gastro-intestinal diseases. Induction of antigen specific oral tolerance (OT) is an attractive therapeutic approach, because it generally lacks toxicity, can be easily administered over time, and avoids side-effects associated with generalized immune suppressive intervention. Oral administration of (auto)antigens or allergens can effectively prevent the induction of autoimmune and allergic diseases in animal models, but several clinical
attempts to induce OT for therapeutic purposes have failed. These failures may be related to the source, the purity, and the amount of (auto) antigen needed, or to the way the antigen is presented to the mucosal immune system. Experimental data indicate that heterogeneous antigen mixtures are less effective inducers of OT than single purified antigens, and that the antigen dose is critical. High-doses of antigen can lead to clonal deletion or anergy of the T cells recognizing the antigen, whereas low doses can induce active suppression, e.g. by inducing antigen-specific regulatory T cells secreting suppressive cytokines. 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. Different types of regulatory T cells can be induced or expanded by mucosal antigens, including CD4+CD25+, CD4+CD25−, and CD8+ regulatory T cells through a TGF-β and/or IL-10 dependent mechanism.

The Lactococcus lactis (L. lactis)-mediated delivery system obviates the need for large-scale purification of human autoantigens or allergens, and enables delivery of antigens to the intestinal mucosa. L. lactis is a non-pathogenic, non-invasive, noncolonizing gram-positive bacterium, and according to the US Food and Drug Administration it is Generally Regarded As Safe (GRAS). Previously genetically modified L. lactis strains have been produced for synthesis and delivery of immunomodulatory proteins at the intestinal mucosa, and an adequate biological containment system has been established. A biologically contained L. lactis strain secreting human IL-10 was used in a phase I, open label clinical trial on Crohn’s disease patients. This trial demonstrated that treatment of humans with viable L. lactis secreting IL-10 is clinically and biologically safe, and gave indications of its clinical efficacy. The use of genetically modified L. lactis for intestinal delivery of proteins in humans warrants further investigation.

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

Methods

Bacteria and media

The *Lactococcus lactis* MG1363 (LL) strain\(^{17}\) was genetically modified and used throughout this study. Bacteria were cultured in GM17E medium consisting of M17broth (Difco Laboratories, Detroit, MI) supplemented with 0.5% glucose and 5 µg/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 they reached a saturation density of 2x10^9 colony forming units (CFU) per ml. Bacteria were harvested by centrifugation and resuspended in BM9 medium at 2x10^{10} bacteria/ml. Each mouse received 100 µl of this suspension daily through an intragastric catheter.

Plasmids

The mRNA sequence encoding *Gallus gallus* Ovalbumin was retrieved from Genbank (accession number AY223553) and from published data\(^{18}\). Total RNA was isolated from chicken uterus and cDNA was synthesized using 2 µg total RNA, 2 µM oligo dT primers (Promega Corporation Benelux, Leiden, The Netherlands), 0.01 mM DTT (Sigma-Aldrich, Zwijndrecht, The Netherlands), 0.5 mM dNTP (Invitrogen, Merelbeke, Belgium), 20 U Rnasin (Promega Incorporation Benelux) and 100 U superscript II reverse transcriptase (Invitrogen) in a volume of 25 µl. An OVA cDNA fragment was amplified by Polymerase Chain Reaction (PCR) using the following primers: forward 5’-GGCTCCATCGGTGCAGCAAGCATGGAATT-3’ and reverse 5’-ACTAGTTAAGGGGAAACACATCTGCCAAGAAGAGAA-3’. Reaction conditions were 94°C for 2 min followed by 30 cycles at 94°C for 45 seconds, 62°C for 30 seconds and 72°C for 90 seconds. The amplified fragment was fused to the Usp45 secretion signal\(^{19}\) of the erythromycin resistant pT1NX vector, downstream of the lactococcal P1 promotor\(^{20}\). MG1363 strains transformed with plasmids carrying OVA cDNA were designated *L. lactis* secreting OVA (LL-OVA). The *L. lactis*-pT1NX, which is MG1363 containing the empty vector pT1NX, served as control (LL-pT1NX)\(^{21}\).
Mice

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

Antigen and Antibodies

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

In vitro and in vivo quantification of OVA secreted by L. lactis

Female BALB/c mice were fed 10 serial inoculates of 2x10^9 CFU of LL-OVA in 100 µl BM9 medium, or 100 µl BM9 medium alone (control), once every 30 min. One hour after the final inoculation, intestinal segments were homogenized in PBS containing 1% fetal calf serum (FCS) for analyses. OVA production was quantified by ELISA, and live L. lactis bacterial count by plating tenfold dilutions of the homogenates on GM17E agar plates containing 5 µg/ml erythromycin. OVA secreted in vivo was quantified by sampling the proximal and distal small intestines, the caecums and the colons in two different ways: (i) samples of the entire intestine, including luminal content, and (ii) extensively washed intestinal tissue samples for measurement of mucosal OVA concentrations. Samples were homogenized and OVA was quantified by ELISA. OVA was captured from the saturated culture supernatant by immobilized polyclonal rabbit anti-OVA antibody (2 µg/ml, Research Diagnostics Inc), quantified by anti-OVA biotin-conjugated polyclonal rabbit antibody (2.5 µg/ml, Research Diagnostics Inc) and stained with horseradish peroxidase-conjugated streptavidin (1/1000, BD Pharmingen, San Diego, USA) followed by TMB substrate (BD Pharmingen).
Oral feeding and DTH (delayed-type hypersensitivity)

DO11.10 mice were immunized by s.c. injection of 100 μg OVA in 50 μl of a 1:1 mixture of CFA (Difco, BD, Alphen aan de Rijn, The Netherlands) and saline solution at the base of the tail on the first day\textsuperscript{22}. Mice were fed purified OVA dissolved in 100 μl BM9 as follows: 200 mg on day 8, 1 mg on days 1-5, 1 μg on days 1-5 and 8-12. LL-OVA or LL-pT1NX were administered on days 1-5 and 8-12. Control mice received only BM9. Antigen or bacterial suspensions were introduced into the stomach using an 18-gauge stainless animal feeding needle. Eleven days after sensitization, antigen-specific DTH responses were assessed by injection of OVA. Twenty-four hours later DTH measurements were performed, or spleen and lymph nodes were harvested and the cells assessed for OVA-specific proliferation and cytokine production. For measurement of antigen-specific DTH responses, mice were challenged with 10 μg OVA in 10 μl saline in the auricle of one ear and 10 μl saline in the other. Ear swelling, defined as the increase in ear thickness due to challenge, was measured in a blinded fashion 24 h after challenge using a micrometer (Mitutoyo, Tokyo, Japan). DTH responses were expressed as the difference in swelling between the OVA-injected and the saline-injected ears (swelling due to OVA minus swelling due to saline).

Cell cultures, and proliferation and cytokine assays

Single cell suspensions of spleen and lymph nodes were prepared by passing the cells through 70-μm cell strainers (Becton/Dickinson Labware). Erythrocytes in the spleen cell suspensions were lysed by incubation with red cell lysis buffer. For IL-10 measurements, 1x10\textsuperscript{5} lymphocytes isolated one day after DTH challenge were incubated in triplicate 96-well plates (Costar, Cambridge, USA) in 200 μl complete medium. They were either left unstimulated or were stimulated in the presence of plate-bound anti-CD3 (1:30 concentration; clone 145.2C11, a gift from Dr. R. Mebius) and soluble anti-CD28 (1:1000 concentration; BD Biosciences) for 48 h or with 500 μg/ml OVA for 40 h.

CD4\textsuperscript{+} T cells and CD4\textsuperscript{+}CD25\textsuperscript{-} T cells were enriched using CD4\textsuperscript{+} T cell isolation kit or CD4\textsuperscript{+}CD25\textsuperscript{+} Regulatory T cell isolation kit, and midiMACS columns (all materials from Miltenyi Biotec, Germany).

A T cell proliferation assay was performed using DO11.10 CD4\textsuperscript{+} T cells and bone marrow (BM)-derived CD11c\textsuperscript{+} DCs cultured with LL-OVA or LL-pT1NX. BM-derived DCs were generated as described\textsuperscript{23} and positively selected using anti-CD11c magnetic microbeads and MACS columns (Miltenyi Biotec, Utrecht, The Netherlands) according to manufacturers’
instructions. The CD11c+ cells were plated at 2x10⁶ per well (24-well; Costar, Cambridge, MA, USA) with 4x10⁵ CFU L. lactis or with 1 µg or 1 mg OVA per ml in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamax, 0.1 mM nonessential amino acids (NEAA), 0.4 M sodium pyruvate, 25 mM HEPES (all four from Invitrogen), 50 µM beta-mercaptoethanol (2-ME), and 5 µg/ml erythromycin (Abbott). After 4 h incubation, the bacteria were killed by adding 75 µg/ml gentamycin. Twenty hours later, cells were harvested. 2x10⁵ CD4+ T cells were then cultured with the harvested DCs cells at ratios of 1/0.3, 1/0.11, 1/0.03, and 1/0.01, respectively. The cultures were grown in 96-well U-bottom plates (Becton Dickinson, Alphen aan de Rijn, The Netherlands) in a total volume of 200 µl complete medium consisting of RPMI-1640 with 10% FCS, 2 mM L-glutamax (Invitrogen), 0.1 mM NEAA, 0.4 M sodium pyruvate, 25 mM HEPES, 50 µM 2-ME, 10 U/ml penicillin (Invitrogen) and 10 µg/ml streptomycin (Invitrogen).

To assay proliferation of total splenocyte populations, 2x10⁵ cells were cultured in 96-well U-bottom plates in a total volume of 200 µl complete medium either alone or with OVA, and either with or without anti-IL-10 neutralizing monoclonal antibody (1 µg/ml, clone JES052A5, R&D Systems, Abingdon, UK). OVA was added at concentrations ranging from 1.2 to 100 µg/ml. The anti-IL-10 antibody was added at 1.0, 0.1 and 0.01 µg/ml. To assay proliferation of CD4+ T cells and CD4+CD25− T cell populations, 0.2x10⁵ cells CD4+ T cells or CD4+CD25− T cells were cultured in 96-well U-bottom plates with 1x10⁵ irradiated CD4− cells, acting as antigen presenting cells, and OVA (0 or 100 µg/ml) in a total volume of 200 µl complete medium either with or without TGF-β neutralizing monoclonal antibody (1 µg/ml, clone 1D11, R&D Systems). Cells were cultured for 90 h at 37°C in 5% CO₂ in a humidified incubator. For proliferation assays, 1 µCi/well [³H]-thymidine was added for the last 18 h of culture, DNA was harvested on glass fiber filter mats (Perkin Elmer, Boston, USA), and DNA-bound radioactivity was measured on a scintillation counter (Perkin Elmer). For cytokine measurements, supernatants of the cell cultures used in the different proliferation assays were collected after 72 h of culture and frozen at −20°C. Cytokine production was quantified using the Mouse Inflammation Cytometric Bead Assay (BD Biosciences, Mountain View, CA, USA).

**Flow cytometry analysis**

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

**Adoptive transfer experiments**

Wild-type Balb/c donor mice were immunized by s.c. injection with 100 μg OVA in 50 μl of a 1:1 mixture of CFA (Difco, BD, Alphen aan de Rijn, The Netherlands) and saline solution at the base of the tail on days 0 and 1. On day 7, CD25 subsets isolated from spleens of DO11.10 mice fed with LL-OVA (as previously described) were adoptively transferred in the sensitized Balb/c mice. To obtain the CD25 subsets, spleen cells were stained using the CD4+CD25+ Regulatory T cell isolation kit (Miltenyi Biotec, Germany) and sorted by MACS cell sorting. The CD4+CD25+ and CD4+CD25− populations were sorted again using a FACS diva (B&D), which separated the positive population into CD25_{high} and CD25_{intermediate} subpopulations. Immediately afterwards, 5x10⁴ CD25_{high}, CD25_{int} or CD25− cells in 200 μl PBS were adoptively transferred by i.v. injection into the OVA-immunized Balb/c mice. These mice were then challenged on day 12 with 10 μg OVA in 10 μl saline in the auricle of one ear and 10 μl saline in the other, and DTH responses were measured 24 h later, as described above.

**Statistical analysis**

Results from cytokine measurements are expressed as means ± SEM. Significance of differences between groups in ear-thicknesses and cytokine measurements were tested using one-way ANOVA followed by Student’s t-test. Significance of OVA-specific proliferation was evaluated using a general linear model with repeated measurements. For both tests, statistical significance is indicated as * (p<0.05) or ** (p <0.01).

**Results**

**LL-OVA induces APC mediated T-cell proliferation in vitro**

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

LL-OVA were able to induce BM-DC maturation (data not shown) and proliferation of DO11.10 CD4+T cells that were co-cultured with BM-DC. BM-DC were generated as described in material and methods. 2x10^6 BM-DC were cultivated with 4x10^5 CFU LL-OVA or LL-pT1NX, with 1 µg or 1 mg OVA per ml or with no additive (/). After 4 h, bacteria were killed with gentamycin. Twenty hours later, BM-DC were harvested and their proliferation was assayed using DO11.10 CD4+T cells. 2x10^5 DO11.10 CD4+ T cells were incubated with 6.67x10^5, 2.22x10^5, 7.41x10^4, 2.47x10^4 BM-DC, corresponding to 1/0.33, 1/0.11, 1/0.01 CD4+T cells/BM-DC. Given the considerable higher proliferation after stimulation with 1 mg/ml OVA, this proliferation is shown in a separate graph.

Figure 1 LL-OVA induces APC-mediated T cell proliferation.
L. lactis-derived OVA is bioactive because it induces proliferation of DO11.10 CD4+T cells after pre-incubation of BM-DC with LL-OVA. BM-DC were generated as described in material and methods. 2x10^6 BM-DC were cultivated with 4x10^5 CFU LL-OVA or LL-pT1NX, with 1 µg or 1 mg OVA per ml or with no additive (/). After 4 h, bacteria were killed with gentamycin. Twenty hours later, BM-DC were harvested and their proliferation was assayed using DO11.10 CD4+T cells. 2x10^5 DO11.10 CD4+ T cells were incubated with 6.67x10^5, 2.22x10^5, 7.41x10^4, 2.47x10^4 BM-DC, corresponding to 1/0.33, 1/0.11, 1/0.01 CD4+T cells/BM-DC. Given the considerable higher proliferation after stimulation with 1 mg/ml OVA, this proliferation is shown in a separate graph.

Active intestinal delivery of OVA by L. lactis

To quantify the in vivo secretion of OVA, small intestines, caecums and colons were obtained 1 h after the final inoculation. CFU numbers and OVA concentrations were determined within the entire intestine, including its luminal content (EI) as well as within the intestinal tissue (IT), i.e.
the mucus and mucosa. No live *L. lactis* bacteria were found in the proximal small intestine, whereas in samples of EI from the distal small intestine, the caecum and the colon, CFU averaged 7.25x10⁹, 2.49x10⁹ and 3.49x10⁹, respectively. Relatively few viable *L. lactis* were found in contact with mucus or within the mucosa of the distal small intestine (4.16x10⁶ CFU), caecum (7.03x10⁷ CFU) and colon (1.41x10⁶ CFU) (Table 1). The largest amount of OVA derived from LL-OVA was detected in the colon (48 ng per colon), but only a small fraction of the colon-delivered OVA (3 ng per colon) was found in the intestinal tissue. A smaller amount of OVA was found in the distal part of the small intestine, but this was localized entirely in the mucus and/or mucosa. No OVA was detected in the proximal small intestine (Figure 2). This indicates that *L. lactis* actively

<table>
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<th>CFU LL-OVA</th>
<th>Distal small intestine</th>
<th>Caecum</th>
<th>Colon</th>
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</thead>
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<tr>
<td>Entire intestine, incl. luminal content</td>
<td>7.25 ± 0.14 x 10⁹</td>
<td>2.49 ± 0.15 x 10⁹</td>
<td>3.49 ± 0.015 x 10⁹</td>
</tr>
<tr>
<td>Entire intestine, excl. luminal content</td>
<td>4.16 ± 0.25 x 10⁶</td>
<td>7.03 ± 0.17 x 10⁷</td>
<td>1.41 ± 0.10 x 10⁶</td>
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*Figure 2* OVA is actively produced by LL-OVA in the intestine and is delivered to the small and large intestines. Balb/c mice (n=4) received 10 serial inocula of 2x10⁹ CFU of LL-OVA in 100 µl suspension at intervals of 30 min. One hour after the final inoculation, live bacteria (Tab. 1) and OVA (Fig. 2) were quantified in the entire small intestines, caecums and colons, including their luminal contents (black bar), and in the intestinal tissue (white bar), as described in material and methods. Detection limit of the ELISA = 1.5 ng. Results are representative of two individual experiments.
produced OVA \textit{in vivo}, that was mostly recovered from the mucus and/or mucosa of the small bowel and in the lumen of the large intestine.

\textbf{Administration of LL-OVA significantly suppresses OVA-induced DTH}

OVA-specific DTH reactions in the ear are accurately reflected in ear swelling\textsuperscript{24, 25}. Using an intragastric catheter, OVA-immunized mice were fed BM9 (as a negative control), 200 mg OVA on day 8, 1 mg OVA on days 1-5, 1 µg OVA on days 1-5 and 8-12, or LL-pT1NX (as vector control) or LL-OVA on days 1-5 and 8-12. On day 11, mouse ears, after measurement of thickness, were injected with 10 µg OVA. Ear thickness was measured again after 24 h, and swelling was measured as the increase in ear thickness. Control mice were significantly sensitized to OVA, as evidenced by ear swelling, but daily intra-gastric administration of LL-OVA significantly reduced this swelling (15x10^{-2} mm vs 1x10^{-2} mm, respectively p=0.0152) (Figure 3). Ear swelling was also somewhat reduced in LL-pT1NX-treated mice compared to controls (BM9) (6.5x10^{-2} mm vs 15x10^{-2} mm). Remarkably, mice that were fed either a low dose of

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Oral feeding of LL-OVA significantly reduces DTH responses. DO11.10 mice were sensitized by s.c. injection of 100 µg OVA in CFA on day 1. Mice were orally treated with 200 mg OVA on day 8, 1 mg OVA on days 1-5, 1 µg OVA at days 1-5 and 8-12, or with LL-OVA or LL-pT1NX on days 1-5 and 8-12. Control mice received BM9. On day 11, mice were challenged with 10 µg OVA in 10 µl saline in the auricle of one ear and with 10 µl saline in the other. DTH responses are expressed as the mean difference in ear swelling between the OVA-injected and the saline-injected ears. Results summarize data of 4 independent experiments including 6 mice per group per experiment.}
\end{figure}
**Figure 4** OVA-specific increase in IL-10 production after restimulation of cervical lymph node, spleen and GALT cells.

IL-10 secretion of pooled cervical lymph node cells was determined in the supernatants 24, 48 and 72 h after restimulation. Data are represented as means of IL-10 secretion in pg/ml of at least two separate experiments (**a**). IL-10 production by bulk spleen and GALT cells was measured after *ex vivo* restimulation. Mice were fed BM9, 1µg OVA, LL-pT1NX or LL-OVA as described, and DTH was determined. Thereafter, bulk cell populations were isolated and 1x10^5 cells were restimulated *ex vivo* with 0.5 mg/ml OVA (**b**). To determine antigen specific IL-10 secretion, cells were restimulated with anti-CD3/anti-CD28 (**c**). Data summarize three independent experiments including 6 mice per experiment.
OVA (10x1 μg), comparable to the amount of OVA secreted by the LL-OVA, or a high dose (1x200 mg or 5x1 mg), did not show a significant reduction in swelling compared to the control group. These data strongly indicate that LL-OVA can suppress systemic T cell responses in DO11.10 mice, and that this effect is in part due to the apparent intrinsic tolerogenic effect of *L. lactis*.

**Reduction of DTH response by treatment with LL-OVA is accompanied by an OVA-specific increase in IL-10 production.**

Immediately after measuring DTH, cervical lymph node cells draining the ears of OVA-challenged mice were pooled and restimulated with OVA. This led to higher IL-10 production in the LL-OVA treated mice than in the BM9 (control) and LL-pT1NX mice (Figure 4a). IL-10 production by isolated bulk spleen and gut-associated lymph node cells (GALT) following 40 h *ex vivo* OVA restimulation was also assessed immediately after DTH measurement. Production of IL-10 was significantly higher in restimulated bulk spleen cells from LL-OVA treated mice than in the negative control and in the groups receiving 1 µg OVA or LL-pT1NX. Similar results were obtained using restimulated GALT cells (Figure 4b). The observed production of IL-10 was antigen-specific, because no differences in IL-10 production were observed after restimulation with anti-CD3/anti-CD28 (Figure 4c).

In summary, we found that the production of IL-10 in OVA-stimulated cervical lymph nodes, splenocytes and GALT cells was significantly higher in mice that were treated with LL-OVA.

**Oral feeding with L. lactis suppresses the OVA-specific proliferative capacity of splenocytes and decreases IFN-γ production.**

Analysis of OVA-specific proliferation of splenocytes was used to examine peripheral immune responses. Bulk splenocytes of mice treated with BM9, LL-pT1NX or LL-OVA were isolated on day 12, and the OVA-specific proliferative response was assessed. Control mice were clearly sensitized and showed a high proliferative response. Daily intra-gastric administration of LL-OVA significantly reduced the OVA-specific proliferative response (*p*=0.002) (Figure 5). Noteworthy, the OVA-induced proliferative response was also significantly reduced (*p*=0.022) after intra-gastric administration of LL-pT1NX in comparison to the BM9 control group.

The lower proliferative response of spleen cells was accompanied by a significant downregulation of IFN-γ production after *ex vivo* OVA restimulation, both in the LL-pT1NX and the LL-OVA treated mice (Figure
These data indicate that LL-OVA and LL-pT1NX treatments are both able to suppress systemic T cell responses in DO11.10 mice. Feeding with LL-OVA suppresses the OVA-specific proliferative responses of CD4+ splenic T cells mediated by TGF-β and CD4+CD25- T cells.

To determine whether the reduced proliferative response of bulk splenocytes was related to reduced responsiveness of the CD4+ T cells, splenic CD4+ T cells from the mice treated with BM9, LL-pT1NX or LL-OVA were isolated on day 12, and OVA-specific proliferative responses were assessed. The OVA-specific proliferative response of splenic CD4+ T cells was lower after LL-OVA treatment than in the BM9 and LL-pT1NX control groups (Figure 6a). Because blocking with neutralizing anti-IL-10 did not reverse the reduction in the proliferative response of bulk splenocytes (data not shown), we investigated whether TGF-β was involved. Blocking with TGF-β neutralizing monoclonal antibody did not significantly alter the

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**Figure 5** Feeding of LL-OVA or LL-pT1NX significantly reduces the OVA-specific proliferation and IFN-γ production of bulk splenocytes. DO11.10 mice were immunized by s.c. injection of 100 µg OVA in CFA on day 1. Mice were treated orally with BM9 (control), LL-OVA or LL-pT1NX on days 1-5 and 8-12. On day 12, bulk splenocytes were isolated and tested for OVA-specific proliferation, which is expressed as the mean cpm at different OVA concentrations, and for IFN-γ production after 72 h ex vivo stimulation with 33 µg/ml OVA (upper panel). Data represent at least three separate experiments, including 4 mice per group per experiment.
proliferative response of the CD4⁺ splenic T cells in the BM9 and LL-pT1NX groups, but reversed the reduction in proliferation in the LL-OVA group. Importantly, the reduction in proliferation due to LL-OVA treatment was maintained after depleting CD4⁺CD25⁺ T cells (Figure 6a). Moreover, the proliferative response of the CD4⁺CD25⁻ T cells in the LL-OVA group
significantly increased after blocking TGF-β with a specific neutralizing monoclonal antibody (p=0.028). These proliferation data are supported by the IFN-γ production data (Figure 6b). To further analyze the induced CD4+CD25- Treg population, we defined the percentage of CD4+CD25- lymphocytes expressing two major Treg markers: Foxp3 and CTLA4. For this purpose we stained the isolated CD4+CD25- T cells from mice treated with BM9, 1ug OVA, LL-pT1NX or LL-OVA on day 12, and gated them on the Foxp3+ and CTLA4+ positive subpopulation. A de novo expansion of CD4+CD25- Foxp3+ and CD4+CD25- CTLA4+ is observed only in the LL-OVA treated group (Figure 6c). This indicates that CD4+CD25- regulatory T cells expressing Foxp3 and CTLA4 are induced following LL-OVA treatment, and that in vitro the CD25- cells mediate OVA-specific tolerance through a TGF-β dependent mechanism.

**CD4+CD25- regulatory T cells induced after LL-OVA treatment can transfer OVA tolerance in vivo.**

To assess the functional activity of the different CD4+CD25 subsets in vivo, we fed DO11.10 mice with LL-OVA as described above, and we adoptively
transferred CD4^+CD25^{high}, CD4^+CD25^{int} and CD4^+CD25^- subsets from their spleens into Balb/c mice sensitized by s.c. injection with 100 µg OVA in CFA on days 0 and 1. Five days thereafter mouse ears were injected with 10 µg OVA and ear thickness was measured 24 h later. Remarkably, only adoptive transfer of the CD4^+CD25^- T cells caused a reduction of DTH (p=0.0317) (Figure 7). Together, these data strongly suggest that intra-gastric administration of OVA-secreting *L. lactis* induces OVA-specific immune tolerance that is mediated by CD4^+CD25^- regulatory T cells.

**Discussion**

Our data indicate that genetically modified *L. lactis* can be used for mucosal delivery of antigens, and that this suppresses local and systemic antigen-specific T cell responses in both antigen-specific and non-specific manners. Antigen-specific suppression induced by OVA-secreting *L. lactis* is mediated by CD4^+CD25^- ‘adaptive’ Treg and seems to be dependent on TGF-β. Importantly, OVA dose feeding alone (either high dose or low dose) was less efficient than LL-OVA in reducing the DTH response in our model, indicating that the mode of mucosal delivery of an antigen to the immune system critically determines subsequent immune responses.

Induction of Treg is a major goal for immunotherapy for autoimmune diseases and several inflammatory diseases, and it can be achieved by exposing the mucosal immune system to low doses of antigen. It has been difficult to apply antigen-specific mucosal tolerance for the treatment of human diseases. Mucosal tolerance depends critically on several factors, including the purity, source, and dose of antigen, and the mode of antigen presentation to the mucosal immune system^26^. Here we propose a novel therapeutic strategy: active intestinal synthesis and delivery of an antigen by genetically engineered *L. lactis*, which obviates the need for large-scale purification of human (auto)antigens and circumvents current issues related to induction of oral tolerance in humans.

The *in situ* fate of the bacteria as well as the distribution of OVA produced by the LL-OVA was determined by multiple intra-gastric administrations to Balb/c mice. Except for the proximal part of the small intestine, viable *L. lactis* were found throughout the intestine, with most bacteria located in the distal part of the small intestine and in the caecum. In a study performed by Droualt et al only 10-30 % of the *L. lactis* administered survived the duodenal transit^27^. Here we show that more than 50 % of the administered *L. lactis* was recovered from the intestine. We believe that the BM9 inoculation buffer used in our experiments partially protects the bacteria against the extreme gastrointestinal conditions. The largest
amounts of OVA were detected in the colonic lumen, with only small amounts in the intestinal tissue. Less OVA was found in the distal part of the small intestine, but much of it was in the intestinal tissue, indicating that the OVA is efficiently taken up by the small intestine. We have not studied uptake of OVA and/or *L. lactis* by the distal small intestine in detail, but it is possible that OVA peptide and/or lactococcal antigens were either taken up by M-cells located in Peyer’s patches or that whole *L. lactis* were directly sampled by intraluminal extensions of mucosal dendritic cells. Interestingly, most viable bacteria and the largest amounts of mucosal OVA were present in the distal small intestine, which is the predominant location of the intestinal sampling DC network. In addition, *L. lactis* might serve as a bioadhesive delivery vehicle that delivers the antigen at the intestinal sampling network or intensifies contact with the mucosa. This would increase the antigen concentration gradient and ensure immediate absorption without dilution or degradation in the luminal fluid. The large difference between the immune responses to oral OVA and *L. lactis*-delivered OVA indicates that these routes may be pivotal for induction of *L. lactis*-mediated oral tolerance.

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

We further demonstrated that both LL-pT1NX and LL-OVA reduce the DTH response and OVA-specific proliferation of bulk splenocytes and splenic CD4+ T cells. This reduction was not observed after feeding OVA protein, and it was much more pronounced in the LL-OVA- than in the LL-pT1NX-treated mice. Moreover, it was only in the LL-OVA treated mice that we observed antigen-specific production of IL-10 by OVA-stimulated cervical lymph node cells, splenocytes and GALT cells, as well as TGF-β dependent OVA-specific CD4+ T cell suppression. Moreover, LL-OVA treatment induced within the CD4+CD25 population a T cell subset that expresses the typical Treg markers Foxp3 and CTLA-4. We also proved that the suppressive activity resident in the CD4+CD25 Treg population could be transferred to sensitized Balb/c mice. These data indicate that *L. lactis*
can condition the mucosal immune system towards tolerance induction, and boost antigen-specific induction of Treg by the co-delivered antigen. Different studies have demonstrated the efficacy of genetically modified *L. lactis* to abrogate Th2-type responses induced in allergic mice models using non-transgenic Balb/c mice. However, in these studies the allergic responses were diminished by the induction of counter-regulatory Th1 immune responses. 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 non-colonizing bacteria (Adel-Patient et al., 2003). Apparently, *L. lactis* has not such a Th1 adjuvant effect in DO11.10 OVA TCR transgenic mice. Recently, we demonstrated that the Th1 adjuvant effect of *L. lactis* observed in non-transgenic Balb/c mice is absent in Balb/c mice fed over a long period with natural *L. lactis* (Snoeck et al. in preparation). *L. lactis* is not a normal constituent of the microflora or diet of laboratory mice. As such, it can be considered as a foreign microorganism in mice. In contrast, *L. lactis* has been extensively consumed by humans and has never been associated with any form of pathology. Its main use lies in the manufacture of fermented milk, vegetable, and meat production. It is therefore granted a GRAS status and thus acceptable to believe that *L. lactis* will not have any Th1 adjuvant effect in humans. Currently, we are evaluating the *L. lactis* delivery vehicle for the induction of antigen-specific immune tolerance in *L. lactis* conditioned non-transgenic Balb/c mice.

Several phenotypically and functionally distinct Treg subsets have been implicated in suppression of intestinal inflammation and induction of oral tolerance. TGF-β has long been known to have a pivotal role in the induction of oral tolerance, both as a secreted cytokine and in the form of a latency-associated peptide (LAP) corresponding to the aminoterminal domain of the TGF-β precursor protein. Moreover, TGF-β dependent Treg are efficiently induced by low doses of antigen. Previously, it has been demonstrated that oral tolerance induced by feeding OVA was only partly blocked by CD25 depletion, but completely abrogated by blocking of TGF-β. Recently it was shown that antigen-specific TGF-β producing Th3 cells drive the differentiation of antigen-specific Foxp3+ regulatory cells in the periphery, and play a crucial role in inducing and maintaining peripheral tolerance. Although TGF-β partially mediates the suppressive effects of several types of Treg, both LAP and (membrane-associated) TGF-β are intimately involved in the suppression mediated by a specific subset of GALT-derived CD4+CD25- Tregs that can induce oral tolerance. These cells suppress the colitis induced by transfer of CD4+CD45RBhigh T cells into SCID mice and they are expanded after oral administration.
of low dose anti-CD3 antibody to mice. Here, we demonstrate that oral administration of OVA-secreting *L. lactis* induces a similar Treg population residing within the CD4<sup>+</sup>CD25<sup>-</sup> compartment, but in contrast to oral administration of anti-CD3 antibodies, the induced regulatory cells are antigen specific and Foxp3 and CTLA-4 positive. The exact mechanism by which intestinal CD4<sup>+</sup>CD25<sup>-</sup> Treg interfere with antigen-induced inflammatory reactions needs to be determined, and different possible mechanisms have been proposed. Effector T cells may be directly antagonized through the induction of immunosuppressive cytokines or by cell-cell contact, i.e. by the activity of membrane-bound TGF-β<sup>53, 54</sup>. Suppression can also result via induction of “secondary” Treg that may depend on IL-10 production<sup>55, 56</sup>. Foxp3<sup>+</sup>CD25<sup>-</sup> Th3 regulatory T cells represent a different cell lineage from thymus-derived CD25<sup>+</sup> Tregs in the periphery, but they may play an important role in their maintenance<sup>57</sup>. Moreover, TGF-β dependent conversion of peripheral CD4<sup>+</sup>CD25<sup>-</sup> T cells into CD25<sup>+</sup> CD45RB<sup>-/low</sup> suppressor cells has also been reported<sup>58</sup>. The mechanism by which CD4<sup>+</sup>CD25<sup>-</sup> cells function needs to be determined, but both orally administered anti-CD3 and LL-OVA-induced CD4<sup>+</sup>CD25<sup>-</sup> regulatory cells clearly can suppress systemically induced inflammatory responses.

Therapeutic induction of Treg is a promising strategy for treating or restoring tolerance in patients suffering from autoimmune diseases. Current strategies for therapeutic induction of antigen-specific suppressor cells face considerable hurdles, and usually require techniques to isolate, handle and transfer adequate numbers of regulatory cells<sup>59-62</sup>. The *L. lactis* antigen delivery system circumvents these problems and effectively induces antigen-specific Treg.

In conclusion, our data demonstrate that mucosal delivery of OVA by genetically modified *L. lactis* induces suppression of local and systemic OVA-specific T cell responses in DO11.10 mice, and that this effect does not depend solely on the secreted OVA, but also on the presence of *L. lactis*. Oral administration of an antigen by *L. lactis* resulted in the induction of systemic tolerance mediated by CD4<sup>+</sup>CD25<sup>-</sup> regulatory T cells that seems to function through a TGF-β dependent mechanism. Importantly, the intestinal delivery system by *L. lactis* is superior to oral administration of soluble antigen, since both low and high dose antigen feeding were not able to diminish the DTH response as significant as LL-OVA treatment in this model. These data indicate that engineered *L. lactis* could be an effective tool for inducing antigen-specific tolerance, with possible application in the treatment of antigen-induced autoimmune diseases.
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Chapter 3


OVA tolerance


Chapter 3


In vitro and in vivo down-regulation of regulatory T cell activity with a peptide inhibitor of Transforming Growth Factor β-1


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Chapter 4

Abstract

Downregulation of CD4+CD25+ T regulatory cell (Treg) function might be beneficial to enhance the immunogenicity of viral and tumor vaccines or to induce breakdown of immunotolerance. Although the mechanism of suppression used by Treg remains controversial, it has been postulated that TGF-β1 mediates their immunosuppressive activity. Here we show that P17, a short synthetic peptide that inhibits TGF-β1 and TGF-β2, developed in our laboratory, is able to inhibit Treg activity in vitro and in vivo. In vitro studies demonstrate that P17 inhibits murine and human Treg-induced unresponsiveness of effector T cells to anti-CD3 stimulation, in a mixed leukocyte reaction or to a specific antigen. Moreover, administration of P17 to mice immunized with peptide vaccines containing tumor or viral antigens, enhanced anti-vaccine immune responses and improved protective immunogenicity against tumor growth or viral infection/replication. When CD4+ T cells purified from OT-II transgenic mice were transferred into C57BL/6 mice bearing subcutaneous EG.7-OVA tumors, administration of P17 improved their proliferation, reduced the number of CD4+Foxp3+ T cells and inhibited tumor growth. Also, P17 prevented development of immunotolerance induced by oral administration of OVA by genetically modified Lactococcus lactis in DO11.10 transgenic mice sensitized by s.c. injection of OVA. These findings demonstrate that peptide inhibitors of TGF-β may be a valuable tool to enhance vaccination efficacy and to break tolerance against pathogens or tumor antigens.

Introduction

During the last years, CD4+CD25+ regulatory T cells (Treg) have been the subject of intense study. This is because their function appears to be critical in the maintenance of peripheral tolerance and regulation of immune responses to non-self antigens. Treg can inhibit activation of other T cells (1) and are needed for protection against autoimmune diseases and prevention of rejection of allogeneic transplants. However, immunoregulatory function of Treg may hinder the induction of immune responses against cancer and infectious agents. Thus, the presence of Treg within tumors may prevent activation of antitumor immune responses favoring tumor growth. This suggests that counteracting Treg activity could evoke effective antitumor immunity (2-5). Treg cells capable of suppressing the in vitro function of tumor-reactive T cells have been found in humans in tumors such as melanoma (6, 7), lung (8), ovary (8, 9), pancreas and breast cancers (10) as well as hepatocellular carcinoma.
Moreover, recent findings suggest that Treg infiltrating neoplastic tissues might be associated with a higher death hazard and reduced survival (7, 9, 12). In infectious diseases, the control exerted by Treg may limit the magnitude of effector T cell responses and may result in failure to control infection. Indeed, it has been shown that some viruses, such as hepatitis B (13), hepatitis C (14-17) and HIV (18-21), may exploit Treg to dampen the antiviral response to favor the persistence of the infection.

Although Treg require antigen exposure to initiate suppressive activity, the effector phase seems to be mediated by an antigen non-specific mechanism (22). The mechanism of suppression by Treg remains controversial, with differences between in vitro and in vivo experiments in terms of the relative contribution of soluble cytokines respect to cell-to-cell contact. In many experimental systems, multiple subsets of Treg seem to function in vivo by secreting immunosuppressive cytokines such as transforming growth factor-β (TGF-β) and IL-10 (23-26). It has been suggested that TGF-β produced by Treg and/or bound to the cell membrane, may mediate suppression of T-cells (24). Moreover, it has been described that CD4+CD25+ cell-mediated suppression of autoimmune or antitumor CD8+ cells requires an intact TGF-β receptor II on the CD8+ cells (27, 28). TGF-β is also important in the homeostasis of Treg since it may contribute to the generation and proliferation of Treg cells (29). In addition, in the context of T-cell receptor (TCR) stimulation, TGF-β is able to convert peripheral CD4+CD25− naïve T cells to CD4+CD25+ Treg via induction of transcription factor Foxp3 (30, 31). These data suggest that inhibition of TGF-β, in particular by small molecules that might penetrate the interface between contacting T cells, would be a valuable tool to inhibit Treg activity and concomitantly foster antiviral or antitumor immunotherapies.

Using a phage-displayed random 15-mer peptide library we have identified a peptide inhibitor of TGF-β1, named P17. This peptide blocks the inhibition activity of TGF-β1 on the growth of Mv-1-Lu cells in vitro and also inhibits TGF-β1-dependent expression of collagen type I mRNA in the liver of mice orally challenged with CCl4 (32). In the present study we show that P17 is able to inhibit Treg in vitro and improve the efficacy of vaccination in vivo. Also, P17 reduces the number of CD4+Foxp3+ T cells and augments proliferation of OTII-derived CD4+T cells after their adoptive transfer in mice bearing EG.7-OVA tumors. Moreover P17 efficiently blocked the induction of antigen-specific oral tolerance induced in vivo after intragastric supplementation of the OVA secreting Lactococcus lactis (LL-OVA) in DO11.10 mice (33). Thus, as we discuss in more detail below, P17
clearly holds promise to boost immune responses by downregulation of CD4⁺CD25⁺ Treg, an effect that can be used to enhance the effectiveness of vaccination.

**Materials and Methods**

**Peptides**
KRIWFIPRSSWYERA (P17) is a peptide inhibitor of TGF-β developed in our laboratory (32), peptide SPSYVYHQF (from now on AH1) is a cytotoxic T cell determinant (TCD) expressed by CT26 cells and presented by H-2Ld MHC-class I molecules (34), peptide p1073 (CVNGVCWTV) from hepatitis C virus NS3 protein containing a TCD presented by HLA.A2.1 class I molecules (35), the H2-Kb-restricted OVA TCD peptide SIINFEKL, and an irrelevant control peptide (AKAVVKTFHETLDCC) from human CD81 molecule were synthesized manually in a multiple peptide synthesizer using Fmoc chemistry as previously described (36). The purity of peptides was above 90% as judged by HPLC.

**Mice**

Female BALB/c and C57BL/6 mice were purchased from IFFA Credo (Barcelona, Spain). A breeding pair of HHD transgenic mice expressing human HLA.A2 molecule was kindly provided by Dr F. Lemonnier (France), and were bred and maintained in pathogen-free conditions. OVA-specific TCR transgenic mice (DO11.10) on a BALB/c background were kindly provided by Dr. J.N. Samson (Vrije Universiteit, Amsterdam, The Netherlands) and bred at the Academic Medical Center, Amsterdam. Eight to ten week old DO11.10 mice were used for the experiments. OVA-specific TCR transgenic mice OT-II and OT-I (C57BL/6 background), were kindly provided by Dr I. Melero (CIMA, Pamplona, Spain). All mice were housed in a conventional animal facility under routine laboratory conditions. All experiments performed followed institutional guidelines and were approved by the institutional ethical committees.

**Cell Culture**

BSC-1 cells (kindly provided by Dr. J.A. Berzofsky, NIH Bethesda, MD) were used for titration of vaccinia virus in ovaries. T2 and P815 cells American Type Culture Collection ATCC (Manassas, VA) were used as target cells in chromium release assays with CTL from HHD or BALB/c mice respectively.
OVA-transfected E.G7-OVA cells (H-2\(b\))(37) and CT26 tumor cells (H-2\(d\)) were purchased from ATCC and used in vivo for tumor protection and treatment experiments. They were cultured in complete medium (RPMI 1640 containing 10% fetal calf serum, 100 U/ml of penicillin, 100 µg/ml of streptomycin, 2 mM glutamine and 50 µM 2-ME. Medium for E.G7-OVA cells also contained 400 µg/ml of G418.

**Purification of regulatory T cells**

Isolation of murine and human CD4\(^+\), CD4\(^+\)CD25\(^+\), and CD4\(^+\)CD25\(^-\) T cells was performed from murine spleen cells or from human peripheral blood mononuclear cells by using murine and human regulatory T cell isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany) respectively and according to manufacturer’s instructions. The purity of the resulting T cell populations was confirmed to be >95% by flow cytometry. Expression of membrane-associated TGF-β1 on the surface of CD4\(^+\)CD25\(^+\) T cells was measured by flow cytometry using anti-LAP(TGF-β1) antibodies (R&D Biosystems).

**RNA extraction and Real Time RT-PCR**

Total RNA extraction from CD4\(^+\)CD25\(^-\) or from CD4\(^+\)CD25\(^+\) T cells was performed using the Nucleic Acid Purification Lysis Solution (Applied BioSystems, Foster City, CA) and the semi-automated system ABI PRISM 6100 Nucleic Acid PrepStation (Applied BioSystems). DNase treatment, reverse transcription and quantitative real time PCR for CTLA4, GITR, Foxp3 and IL-10 were carried out as described (38). mRNA values were represented by the formula: 2\(^{ΔCt}\), where ΔCt indicates the difference in threshold cycle between control (β actin) and target genes.

**In vitro assays for murine or human T regulatory cell function**

Inhibitory activity of murine or human Treg was measured in three different in vitro assays of T cell stimulation. CD25 depleted spleen cells (10\(^5\) cells/well) from BALB/c mice or 10\(^5\) human PBMC were stimulated in vitro with: (i) 0,5 µg/ml of anti-mouse or anti-human CD3 antibody (Pharmingen) respectively, (ii) with 10\(^3\) bone marrow dendritic cells from C57BL/6 mice (prepared as described previously (39)) or with 10\(^5\) human PBMC (from a different donor, to induce a MLR), or (iii) with a specific antigen. To study the effect of Treg on antigen-specific T cell stimulation, 10\(^5\) ovalbumin-specific CD8\(^+\) T cells from T-cell receptor (TCR) transgenic OT-1 mice, were incubated with 10\(^3\) bone marrow dendritic cells (from
C57BL/6 mice) pulsed with SIINFEKL peptide (1µg/ml), whereas 10^5 CD4^+CD25^- effector T cells/well isolated from human PBMC were cultured in the presence or absence of tetanus toxoid antigen (5 limit of flocculation (Lf)/ml). All T cell stimulations were carried out in the presence or absence of 10^4 CD4^+CD25+ Treg and the indicated concentrations of peptide P17 or control peptide were added to the cultures. To assess whether CD4^+CD25+ T cells exert their regulatory function through direct cell contact or through release of soluble factors, we performed a series of transwell experiments. Once purified, CD4^+CD25+ T cells were added at a ratio of 1:10 to autologous CD4^+CD25- T cells seeded at 5 x 10^5/well in the lower chamber of a 24-well plate. CD4^+CD25+ T cells were either cultured in the lower chamber directly in contact with the target cells or in the upper chamber separated from the target cells by a 0.4-µm pore membrane (BD Biosciences Discovery Labware), which allows diffusion of small molecules, such as cytokines, but not of cells. T cell proliferation was tested after 3 days of culture by measuring [methyl-^3H]thymidine incorporation. Briefly, the second day of culture 0.5 µCi [methyl-^3H] thymidine were added to each well and incubated overnight. Cells were harvested (Filtermate 96 harvester; Packard Instrument, Meriden, CT) and incorporated radioactivity was measured using a scintillation counter (Topcount; Packard Instrument). IFN-γ secretion to the culture supernatant was measured by ELISA (Pharmingen, San Diego, CA) according to manufacturer’s instructions.

**In vitro assays of T cell proliferation in the presence of TGF-β1 or TGF-β2**

Recombinant human TGF-β1 or TGF-β2 inhibit proliferation of murine or human derived effector T cells stimulated with anti-CD3. The IC50 (inhibitory concentration 50%) for human TGF-β1 was 20 pg/ml and 1 pg/ml when using murine and human derived effector T cells respectively. For TGF-β2, the IC50 was 0.25 pg/ml when using murine splenocytes. Splenocytes from C57BL/6 female mice, or peripheral blood lymphocytes from human blood donors, were cultured (10^5 cells/well) in the presence or absence of 0.5 µg/ml anti-mouse or anti-human CD3 antibodies and in the presence or absence of the corresponding IC50 of exogenously added human TGF-β1 or human TGF-β2 (R&D Biosystems). P17 or control peptide were added to the cultures at the concentrations indicated in figures. T cell proliferation was tested after 3 days of culture by measuring [methyl-^3H] thymidine incorporation as described above.
**Biomolecular interaction analysis**

Screening of peptide binding to TGF-β1, TGF-β2 and TGF-β3 was performed by surface plasmon resonance (SPR) using a BIAcore X Biosensor (BIAcore, AB, Uppsala, Sweden). TGF-β isoforms (R&D Systems, USA) as well as an irrelevant protein were covalently immobilized onto the surface of flow cell 2 (FC2) of CM5 chips (BIAcore) as described (40). Flow cell 1 (FC1), which does not contain immobilized TGF-β, was used as the reference flow cell. Individual peptide solutions (5 µM) were injected three times in 10 mM Hepes, 150 mM NaCl, 0.005 % (v/v) Tween-20, 0.1 mg/ml BSA, pH 7.4, at a flow of 30 µl/min. Mass transport limitation was excluded. Curves were processed by subtracting the response in FC1 from that in FC2.

**Immunization experiments and measurement of T cell activation**

BALB/c or HHD mice were immunized subcutaneously at day 0 with 50 nanomoles of TCd peptides AH1 or p1073 respectively, emulsified in IFA. Fifty nanomoles/mouse of peptide P17 was administered intraperitonealy (i.p.) at days 6, 7, 8 and 9 after immunization. At day 10, mice were sacrificed, spleens were removed, homogenized and 8 × 10⁵ cells cultured in 96-well plates in complete medium in the presence or absence of 10 nM of the corresponding TCd peptide. Peptide-specific CTL responses were measured using a conventional cytotoxicity assay as previously described (41). IFN-γ produced in response to the TCd was measured by ELISA (Pharmingen) in culture supernatants (50 µl) harvested after 48 hours of culture, according to manufacturer’s instructions.

**Effect of P17 in anti-tumor peptide vaccination**

Animals immunized with 50 nanomoles of peptide AH1 emulsified in IFA as previously described (42), were treated with 50 nanomoles of peptide P17 or with saline at days 6, 7, 8, 9 and 10. Ten days after immunization, mice were challenged by s.c. injection with 5 x 10⁵ CT26 tumor cells. In an independent experiment, 100 µg per dose of neutralizing anti-TGF-β polyclonal antibody from rabbit, or the corresponding isotype control (R&D Systems) were administered i.p. to mice using the same schedule as for P17. Tumor size was monitored twice a week with a caliper and it was expressed according to the formula V= (length x width²)/2. Mice were sacrificed when tumor size reached a volume greater than 4 cm³.
**Effect of P17 in “in vivo” T cell transfer experiments in E.G7-OVA tumor bearing mice**

Groups of five C57BL/6 mice were challenged with \(5 \times 10^5\) E.G7-OVA tumor cells. When tumors reached 12.5 mm\(^3\), mice were adoptively transferred with \(3 \times 10^6\) CFSE-labeled CD4\(^+\) T cells isolated from OT-II transgenic mice. CFSE cell labeling was carried out by incubation with 1 µM CFSE for 10 min at room temperature followed by three washes with PBS. After T cell transfer, a group of mice was treated daily with 50 nanomoles of peptide P17 by i.p. route. Five days after T cell transfer, mice were immunized i.v. with 50 µg of OVA (Sigma, St Louis, MO) in PBS and sacrificed 3 days after immunization. Splenocytes were isolated and the analysis of CFSE-labeled CD4\(^+\) T cell proliferation and CD4\(^+\)Foxp3\(^+\) cell staining was carried out by flow cytometry. Analysis of CD4\(^+\)Foxp3\(^+\) cells was carried out using the mouse regulatory T cells staining kit (from eBioscience. San Diego, CA) according to manufacturer’s instructions. Tumor size was measured the day of sacrifice as described above.

**Effect of P17 in in vivo protection against infection with a recombinant vaccinia virus expressing HCV proteins**

HHD mice immunized with peptide p1073 as described above, were treated i.p. with 50 nanomoles of peptide P17 or with saline at days 6, 7, 8 and 9 after immunization. They were challenged i.p. at day 10 with \(5 \times 10^6\) pfu of the recombinant vaccinia vHCV1-3011 expressing HCV polyprotein. Three days after vaccinia challenge, mice were sacrificed and viral titre measured as described (43).

**Effect of P17 in a model of hypersensitivity**

The *Lactococcus lactis* MG1363 (LL) strain was genetically modified and used throughout this study, as described before (44, 45). Bacteria were cultured in GM17E medium i.e., M17broth (Difco Laboratories, Detroit, MI) supplemented with 0.5% glucose and 5 µg/ml erythromycin (Abbott B.V., Hoofddorp, The Netherlands). Bacteria were diluted 200-fold in GM17E medium, incubated at 30 ºC overnight and harvested by centrifugation and concentrated in BM9 medium at \(2 \times 10^9\) bacteria/100 µl. Treated mice, received 100 µl of this suspension daily by intragastric catheter (46). DO11.10 mice were sensitized by s.c. injection of 100 µg OVA in 50 µl of a 1:1 CFA (Difco, BD, Alphen aan de Rijn, The Netherlands) saline solution in the tail base at day 1 (47). Mice were fed BM9 as a control or LL-OVA.
(both at days 1-5 and 8-12) administrations using a stainless 18-gauge animal feeding needle. Every other day, starting at day 0, mice received 50 nanomoles/mice of P17 peptide. Eleven days after sensitization, antigen-specific DTH responses were assessed. For DTH measurement, mice were challenged with 10 μg OVA in 10 μl saline in the auricle of one ear and 10μl saline in the other. The increase in ear thickness was measured in a blinded fashion using an engineer’s micrometer (Mitutoyo, Tokyo, Japan) at 24 h after challenge. DTH responses were expressed as the difference in increase between the OVA injected and the saline injected ear thickness, following subtraction of ear-thickness before the challenge (DTH response=OVA-saline-baseline). Intact, LPS-free OVA grade V protein was used as antigen (Sigma Aldrich).

For cytokine measurements, 2 x 10^5 cells of splenocytes were cultured in 96-well U-bottom plates in a total volume of 200 µl complete medium with 100 µg/ml OVA. Cells were cultured at 37°C in a 5% CO2 humidified incubator and after 72 h, culture supernatants were collected 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).

**Statistical analysis**

Normality was assessed with Shapiro-Wilk W test. Statistical analyses were performed using parametric (Student’s t test and one-way ANOVA) and non-parametric (Kruskal-Wallis and Mann-Whitney U) tests. For all tests a \( p \) value < 0.05 was considered statistically significant. Descriptive data for continuous variables are reported as means±SD. SPSS 9.0 for Windows was used for statistical analysis.

**Results**

**Peptide P17 inhibits Treg in vitro**

Previous studies have shown that murine CD4^+CD25^+ regulatory T cells produce high levels of TGF-β1 bound to the cell surface and/or secreted to the medium. Blockade of this TGF-β1 by anti-TGF-β may limit the ability of these cells to suppress CD25^- T cell proliferation (24, 48). In a previous work, we showed that P17, a peptide inhibitor of TGF-β1, inhibited TGF-β1-dependent expression of collagen type I mRNA in a model of liver damage (32). In Figure 1A and 2A we show that P17 is also able to inhibit, in a dose dependent manner, the immunosuppressive activity of
TGF-β1 exogenously added to murine or human derived effector T cells stimulated with anti-CD3 (see methods). Inhibitory activity of P17 was similar to that found when 2 µg/ml of neutralizing anti-TGF-β1 antibodies were added to the cultures. To study the capacity of P17 to inhibit the

A

B

C

D

E

F

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suppressor activity of Treg cells *in vitro*, we purified CD4+CD25+ T cells from murine splenocytes and studied their immunosuppressive activity over effector T cells stimulated with anti-CD3 antibodies. Purified Treg had high mRNA levels for CTLA4, GITR, Foxp3 and IL-10 and expressed TGF-β1 bound to latency-associated peptide (LAP) on their membrane (Figure 1B). To assess whether CD4+CD25+ T cells exert their regulatory function through direct cell contact, or through release of soluble factors, we separated effector T cells from Tregs using Transwell assays. It was found that purified Tregs exerted their inhibitory activity only when in direct contact with effector T cells (Figure 1C).

We then tested the effect of P17 in co-cultures of effector T cells and Treg cells isolated either from mouse spleen cells or from human PBMC. In these assays P17 was able to inhibit Treg suppressive function, restoring the proliferation of murine effector T cells or human PBMC in response to soluble anti mouse CD3 or anti-human CD3 antibody respectively (Figures 1D and 2B). Addition of anti-TGF-β neutralizing polyclonal antibody was also able to significantly inhibit Treg activity. P17 was also able to restore proliferation of effector T cells in MLR, when bone marrow derived dendritic cells from C57BL/6 were co-cultured with non-adherent spleen cells from BALB/c mice in the presence of Treg (Fig 1E), or when human PBMC from two different donors were mixed in the presence of human purified Treg (Fig 2C). P17 also reverted Treg mediated inhibition of

**Figure 1.** P17 inhibits immunosuppression caused by TGF-β or by murine T regulatory cells. (A) Inhibition of TGF-β: Spleen cells from BALB/c mice were stimulated with anti-CD3 monoclonal antibodies in the presence or absence of exogenously added recombinant human TGF-β1 (20pg/ml) and the indicated concentration of P17, control peptide (Pcont) or polyclonal anti-TGF-β or the corresponding isotype control antibodies (2 µg/ml). (B) Characterization of purified CD4+CD25+ T cells: Expression of TGF-β1 bound to latency-associated peptide (LAP) on their membrane was measured by flow cytometry, and mRNA expression of Treg associated genes was measured by real time PCR. (C) Immunosuppression caused by purified CD4+CD25+ T cells is contact-dependent: effector T cells were stimulated with anti-CD3 in the presence of purified CD4+CD25+ T cells either in direct contact or separated by a 0.4-µm pore membrane. (D, E and F) Inhibition of murine T regulatory cells by P17: (D) spleen cells from BALB/c mice were stimulated with anti-CD3 monoclonal antibodies in the presence or absence of purified CD4+CD25+ T cells; (E) Mixed leukocyte reaction using spleen cells from BALB/c mice (10^5 cells/well) and bone marrow derived dendritic cells from C57BL/6 mice (10^3 cells/well); (F). T cells from OT-1 mice were cultured with DC pulsed with SIINFEKL peptide, with or without of CD4+CD25+ Treg cells. Different concentrations of P17 (12.5 to 100 µM) were tested in (D) to measure inhibition of Treg activity, whereas a single concentration (100 µM) was tested in (E and F). A concentration of 2µg/ml of polyclonal anti-TGF-β or the corresponding isotype control antibodies (2 µg/ml) was used in (A and D). Cell proliferation (A, C, D, E and F) was analyzed by measuring 3H trititated thymidine incorporation in the harvested cells using a scintillation counter. IFN-γ released to the culture was quantified by ELISA (E). t test *, p<0.05 (comparison between the indicated group (*) and the corresponding control of immunosupression in the absence of inhibitors). The results are representative of at least three different experiments per each panel.
antigen-specific T cell activation in the case of T cells from OT-I transgenic mice responding to peptide OVA (257-264) (SIINFEKL) from ovalbumin (Fig 1F), or in the case of human PBMC responding to tetanus toxoid (Fig 2D). The percentage of inhibition of the Treg effect by 100 µM of P17 was found to vary between 25% and 100% in different in vitro models. This variation may be related to the role of TGF-β in each particular model. In summary, results from Fig 1 and 2 indicate that P17 is able to inhibit, at least partially, both murine and human Treg in vitro.

**Figure 2.** *P17 inhibits immunosuppression caused by TGF-β or by human T regulatory cells.*

(A) Inhibition of TGF-β1: Human PBMC were stimulated with anti-CD3 monoclonal antibodies in the presence or absence of exogenously added recombinant human TGF-β1 (1pg/ml) and the indicated concentration of P17 or polyclonal anti-TGF-β or the corresponding isotype control antibodies (2 µg/ml). (B, C and D) Inhibition of human Tregs: (B) Human PBMC were stimulated with anti-CD3 monoclonal antibodies in the presence or absence of recombinant human TGF-β1 (1pg/ml) and the indicated concentration of P17 or polyclonal anti-TGF-β or the corresponding isotype control antibodies (2 µg/ml). (C) Mixed leukocyte reaction using PBMC isolated from two donors; (D) Inhibition of human Tregs: (D) Human PBMC were cultured with or without tetanus toxoid in the presence or absence of human CD4+CD25+ Treg. Different concentrations of P17 (12 to 100 µM) were tested in (B) to measure inhibition of Treg activity, whereas a single concentration (100 µM) was tested in (C and D). Cell proliferation (A to D) was analysed by measuring 3H trititated thymidine incorporation in the harvested cells using a scintillation counter. t test *, p<0.05 (comparison between the indicated group (*) and the corresponding control of immunosuppression in the absence of inhibitors). The results are representative of at least three different experiments per each panel.
**Peptide P17 also inhibits the immune-suppressive activity of TGF-β2 isoform**

The forkhead transcription factor Foxp3 is highly expressed in CD4⁺CD25⁺ Tregs and act as a key player in mediating their inhibitory functions. But also, a recent paper has described that tumor cells can be induced to express functional Foxp3 by TGF-β2, in such a way that naïve T-cell proliferation is inhibited when cocultured with these Foxp3-expressing tumor cells (49). Since TGF-β isoforms have a high homology (70-80%), we investigated the capacity of P17 peptide to bind to these isoforms by Surface Plasmon Resonance. As shown in Figure 3A, binding of P17 follows the order TGF-β1>TGF-β2>>>TGF-β3. As expected, P17 was unable to bind to an irrelevant protein (Fig 3A) and a control peptide from human CD81 molecule did not bind to any of the TGF-β isoforms (data not shown). Since P17 was able to bind TGF-β2, we tested its ability to inhibit the immunosuppressive activity of this cytokine *in vitro*. Thus, we added TGF-β2 to effector T cells stimulated with anti-CD3 (see methods). It was found that 100 µM of P17 inhibited the immunosuppressive activity of TGF-β2 by around 50% (Fig 3B).

![Figure 3](image-url)

**Figure 3.** *P17 binds to TGF-β2 and inhibits its immunosuppressive activity.*

(A) Binding assays: TGF-β1, TGF-β2, TGF-β3 or an irrelevant protein were immobilized covalently on the flow cell FC2 of a CM5 chip via a standard amine coupling procedure. P17 solution (5 µM) was injected three times over the protein bound surfaces. Sensograms of P17 binding capacity to the different TGF-β isoforms or to the irrelevant protein are plotted. (B) Inhibition of TGF-β2 activity: Spleen cells from BALB/c mice were stimulated with anti-CD3 monoclonal antibodies in the presence or absence of exogenously added recombinant human TGF-β2 (0.25pg/ml) and 100 µM of P17 or control peptide (Pcontrol). Cell proliferation was analyzed by measuring ³H tritiated thymidine incorporation in the harvested cells using a scintillation counter. *t* test *, p<0.05 (when compared T cell proliferation in the presence versus the absence of P17). The results are representative of two independent experiments.
Peptide P17 improves immunogenicity of AH1 peptide vaccination leading to protection against CT26 tumor challenge

Downregulation of Treg suppressor activity in vivo might be beneficial to enhance vaccine immunogenicity against tumor antigens. Immunization of BALB/c mice only with peptide AH1 (a TCd, expressed by CT26 colon cancer cells) is unable to induce a protective CTL response against challenge with CT26 tumor cells (34). However, this result could be overcome by co-immunization of AH1 and an adequate T helper cell determinant capable of inducing a competent T helper response (42). We have also shown that depletion of CD25+ Treg cells with anti-CD25 antibodies before immunization with peptide AH1, permits the induction of a long lasting anti-tumoral immune response (2). All these observations lead us to speculate that in vivo inhibition of Treg by peptide P17 (instead of Treg depletion) in combination with vaccination with AH1, might allow the control of tumor growth. Thus, BALB/c mice were immunized with peptide AH1 emulsified in IFA at day 0 and treated with saline or with 50 nanomoles/mice of peptide P17 at days 5, 6, 7, 8 and 9 after immunization. As shown in Figure 4A, immunization with AH1 does not induce IFN-γ producing cells specific for AH1 peptide. However, in vivo treatment of AH1 immunized mice with P17 strongly augments AH1 immunogenicity.

Figure 4. Peptide P17 improves immunogenicity of AH1 peptide vaccination leading to protection against CT26 tumor challenge. Groups of BALB/c mice were immunized with saline (n=10) or, with peptide AH1 emulsified in IFA (n=20). Ten mice immunized with AH1 were treated i.p. with saline at days 5, 6, 7, 8 and 9 after immunization, whereas the remaining 10 mice were treated with 50 nanomoles/mice of peptide P17. At day 10 after immunization, two mice from AH1 immunized groups were sacrificed and spleen cells were cultured in the presence or absence of peptide AH1. After 48 hours of culture, supernatants were removed and IFN-γ released to the culture was quantified by ELISA (A). The remaining 8 mice from both groups of AH1-immunized mice (the one treated with AH1 only and the other treated with AH1 + P17) as well as mice injected with saline were challenged s.c with 5 x 10⁵ CT26 tumor cells. Tumor size was measured as described in Methods. Mice were sacrificed when tumor size reached a volume greater than 4 cm³. The ratio 6/8 indicates that 6 mice (out of the 8 treated with AH1 + P17) remained tumor free (B). The results are representative of two different experiments.
Moreover, when mice were challenged s.c. with $5 \times 10^5$ CT26 tumor cells, six out of eight mice immunized with AH1 and treated with peptide P17, remained tumor-free, whereas all mice immunized with AH1 only or left unimmunized developed tumors (Figure 4B). These results indicate that TGF-β inhibition by P17 improves immunogenicity of AH1 peptide vaccination and concomitantly protection against tumor growth.

**Peptide P17 reduces the number of CD4+Foxp3+ cells and improves T cell proliferation of OT-II derived CD4+ T cells adoptively transferred to mice bearing E.G7-OVA tumors**

A number of tumor cells have been shown to produce TGF-β both in vitro and in vivo, which may mediate immunosuppression in the hosts (50, 51). TGF-β1 is important in maintaining functional Foxp3+ CD4+CD25+ Treg cells and can also induce Foxp3 expression in naïve T cells (30, 31, 52). We therefore wanted to test the in vivo effect of P17 on the number of CD4+Foxp3+ T cells after the adoptive transfer of CFSE-labeled CD4+ T cells.

![Figure 5](image.png)

**Figure 5.** Peptide P17 reduces the number of CD4+Foxp3+ cells and improves T cell proliferation of OT-II derived CD4+ T cells adoptively transferred to mice bearing E.G7-OVA tumors. C57BL/6 mice bearing E.G7-OVA tumor were adoptively transferred with $3 \times 10^6$ CFSE-labeled CD4+ T cells purified from OT-II mice. At day 10 after adoptive transfer, mice were treated daily with PBS (panels A and C) or with peptide P17 (panels B and D) ($n=5$ mice per group). All mice were immunized with OVA protein at day 5 after transfer and sacrificed 3 days later to evaluate proliferation of transferred CD4+ T cells in the spleen (panels A and B) or the numbers of splenic CD4+Foxp3+ T cells (panels C and D) by flow cytometry. Tumor size was measured the day of sacrifice as described in Methods. Results are representative of two different experiments.
cells from OT-II transgenic mice to animals bearing subcutaneous E.G7-OVA tumors. E.G7-OVA cells are derived from EL-4 thymoma cell line, which produces high amounts of TGF-β \textit{in vitro} and \textit{in vivo} ((53), and data not shown). Thus, 16 mice were injected with 5 x 10^5 E.G7-OVA tumor cells, and once the tumors reached 12.5 mm^3, mice were adoptively transferred with 3 x 10^6 CFSE-labeled CD4^+ OT-II derived T cells. After adoptive transfer, mice were treated daily with PBS or with 50 nanomoles of peptide P17 \((n=8\) mice per group). All mice were immunized with OVA protein at day 5 after adoptive transfer, and sacrificed 3 days thereafter to evaluate proliferation of the transferred CD4^+ T cells. As shown in Figure 5, P17 treatment improves proliferation of adoptively transferred CD4^+ T cells \((16.5\%\) of undivided OVA specific CD4^+ T cells (panel B) \textit{vs} 38.7 \% in PBS treated mice (panel A)). This improved T cell proliferation in P17-treated mice, was accompanied by a reduction of the total numbers of CD4^+Foxp3^+ T cells (compare panels D (P17 treated) and panel C (PBS-treated)). Improved proliferation of CD4^+ transferred T cells and reduction in the number of CD4^+Foxp3^+ cells was associated with a diminution in tumor size at the day of sacrifice \((p<0.05\), Figure 5E).

**Peptide P17 improves immunogenicity of p1073 peptide vaccination and protects mice against challenge with recombinant vaccinia vvHCV 3011 virus**

As described above, Treg may hamper the induction of protective cellular immune responses in several viral infections. In particular, it has been recently reported that patients with chronic hepatitis C have a higher number of peripheral Treg, suggesting that these cells might play a role in the chronicity of infection (14-17). We tested the capacity of P17 administration to improve immunogenicity of an HCV-NS3 derived peptide. HHD transgenic mice were immunized s.c with peptide p1073, encompassing a HLA.A2.1 restricted epitope from HCV NS3 protein, emulsified in IFA. At days 5-9 after immunization, mice were treated i.p. with 50 nanomoles/mice of P17 or with saline. Ten days after immunization, animals were sacrificed and spleen cells were cultured with p1073 for five days. Lytic activity was measured in a conventional chromium release assay using T2 cells pulsed with peptide 1073. It was found that P17 treatment of immunized animals was able to prime a cytotoxic T cell response specific for peptide p1073, which was not elicited in animals immunized with p1073 and treated with saline instead of P17 (Figure 6A). The ability of P17 treatment to improve immunogenicity of p1073 was also measured \textit{in vivo}, based on its capacity to protect mice against challenge with a recombinant vaccinia virus expressing the whole polyprotein of
Breakdown of immunotolerance by P17

We have recently demonstrated that mucosal delivery of OVA by genetically modified Lactococcus lactis (LL-OVA) induces suppression of local and systemic OVA specific T-cell response in DO11.10 mice that is mediated by the induction of OVA-specific CD4+CD25- Treg which are critically depend on TGF-β (33). In order to further validate the ability of P17 in this model, mice were sensitized by s.c. injection of 100 µg of hepatitis C virus, as a surrogate of HCV infection. Thus, HHD transgenic mice were immunized s.c. with saline (n=12) or with p1073 emulsified in IFA (n=12). At days 5-9 after immunization half of the mice from each group were treated i.p. with P17. Ten days after immunization, mice were challenged i.p. with 5 x 10^6 pfu of the recombinant vaccinia vvHCV 3011 expressing the HCV polyprotein. Three days after challenge, ovaries were harvested and vaccinia titer was measured by plating on BSC-1 cells. Viral load is expressed as pfu per mg of ovary. Bars represent the mean average of virus titers from 6 mice ±SEM. Results are representative of two different experiments.

**Figure 6.** Peptide P17 improves immunogenicity of p1073 peptide vaccination and protects mice against challenge with recombinant vaccinia vvHCV 3011 virus expressing HCV polyprotein. (A) HHD transgenic mice were immunized with peptide p1073 from HCV NS3 protein emulsified in IFA and treated i.p. with saline or with fifty µg/mice of peptide P17 at days 5, 6, 7, 8 and 9 after immunization. Ten days after immunization, mice were sacrificed and spleen cells were cultured in the presence or absence of peptide p1073. After 5 days of culture, CTL activity against peptide-loaded T2 target cells was measured in a conventional Chromium release assay. (B) Groups of HHD transgenic mice were immunized with saline (n=12) or with peptide p1073 from HCV NS3 protein emulsified in IFA (n=12). Six mice per group were treated with P17 as described in (A). Mice were challenged i.p. with 5 x 10^5 pfu of the recombinant vaccinia vvHCV 3011 expressing the HCV polyprotein. Three days after challenge, ovaries were harvested and vaccinia titer was measured by plating on BSC-1 cells. Viral load is expressed as pfu per mg of ovary. Bars represent the mean average of virus titers from 6 mice ±SEM. Results are representative of two different experiments.
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OVA in 50 μl of a 1:1 CFA saline solution in the base of the tail, and oral tolerance was induced with a 10-day oral administration of LL-OVA. Six of the 12 LL-OVA treated mice received 50 nanomoles of peptide P17 in PBS by i.p. route at alternate days until the end of the experiment. LL-OVA treated mice were significantly tolerized compared to the control mice (1.4 x 10^-2 mm 15.8 x 10^-2 mm). Co-injection of P17 blocked the induction of antigen specific oral tolerance measured by a significant increase in ear-thickness compared to the LL-OVA treated mice (11 x 10^-2 mm vs. 1.4 x 10^-2 mm) (Fig 7A). Immediately after DTH measurements, spleens were isolated and ex vivo stimulated with OVA for 72 hours. In agreement with this finding, it was found that P17 treatment restored IFN-γ production by splenocytes in response to OVA antigen, a response which was totally inhibited by LL-OVA administration (214.4 vs 15.1 pg/ml, respectively, Fig 7B). These results indicate that in this model, administration of P17 effectively interferes with the development of antigen-specific immunotolerance.

Figure 7. In vivo injection of P17 inhibits the induction of oral tolerance by OVA secreting Lactococcus lactis and increases the IFN-γ production of bulk splenocytes. DO11.10 mice were sensitized by s.c. injection of 100 µg OVA in CFA at day 1. Mice were orally treated with BM9 (control) or with LL-OVA at days 1-5 and 8-12. Every other day one LL-OVA treated group received i.p. injection of P17. At day 11, mice were challenged with 10 µg OVA in 10 µl saline in the auricle of one ear and 10 µl saline in the other. DTH responses are expressed as the mean differences in ear thickness increase between the OVA injected and saline injected mice, following subtraction of ear-thickness before OVA challenge (A). All groups consisted of 6 mice. On day 12, bulk splenocytes were isolated and tested for IFN-γ production after 72-hour ex vivo stimulation with 100µg/mL OVA (B).
Discussion

Naturally occurring Treg cells inhibit T-cell proliferation *in vitro*, a mechanism which plays the beneficial role of controlling T-cell responses to self-antigens. This prevents the development of autoimmune diseases (54, 55) as well as the induction of harmful immune responses after organ transplants (55). However, Treg cells may also limit the magnitude of effector responses, which although under certain circumstances may reduce collateral tissue damage caused by vigorous antimicrobial immune responses, may result in failure to adequately control infections (56). Moreover, Treg might hinder the induction of immune responses against cancer (57). All these observations suggest that adequate control of Treg activity may have important implications in medicine.

Accumulating evidences support the role played by TGF-β as a mediator of Treg *in vitro* and *in vivo* (54). Thus, TGF-β directly inhibits proliferation and acquisition of effector function of naïve T cells. In the absence of TGF-β signaling in T cells, the dominant negative TGF-β receptor type II (dnTβRII) transgenic mice develop a lymphoproliferative syndrome and autoimmunity (58-60), probably because their T cells escape control by Treg (58). Moreover, it has been described that TGF-β produced by Treg and/or bound to the cell membrane may mediate suppression of T-cells (24). Also, recent data shows that dendritic cells may inhibit T cell activation via the secretion of TGF-β (61) or by surface expression of TGF-β bound by latency-associated peptide LAP (62). TGF-β is also important in the homeostasis of Treg since it may contribute to their generation and proliferation (29-31, 63). All these data suggest that inhibition of TGF-β, in particular by small molecules that might penetrate the interface between contacting T cells, might be useful to potentiate antiviral or antitumor immunotherapies.

We have shown that P17, a TGF-β inhibitor peptide developed in our laboratory (64), is able to inhibit murine or human derived Treg activity *in vitro* in three different experimental settings. Thus, P17 was able to restore murine or human T cell proliferation in response to anti-CD3 stimulation, which was inhibited by the addition of Treg. Similarly, P17 restored, at least partially, T cell proliferation in a MLR inhibited by Treg. P17 also inhibited Treg cells activity over specific T cells stimulated by an antigen. These results prompted us to test P17 *in vivo*. In a previous work we showed that in vivo CD25⁺ T cell depletion improved immunogenicity of AH1 peptide in vaccination and protected mice against tumor challenge (2). We found that in vivo P17 administration, instead Treg depletion,
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was also able to enhance immunogenicity of AH1 peptide vaccination, and protected mice from CT26 tumor challenge. Downregulation of Treg suppressor activity in vivo may be beneficial to enhance immunogenicity of a vaccine (2). Similarly, P17 administration improved the immunogenicity of a peptide vaccine consisting in the immunization of peptide p1073, which encompasses a HLA.A2.1 restricted epitope from HCV NS3 protein, the outcome being a reduction of recombinant vaccinia vvHCV 3011 virus replication after vaccination with p1073. These results are in agreement with previous reports showing an enhancement of immunogenicity of a vaccine by the depletion of Treg (2-5, 65). However, we believe that in vivo inhibition of Treg activity by P17, instead of Treg depletion, might allow a better control of Treg function, reducing the risk of autoimmune diseases that may be favored in the absence of Treg (66). When we compared the effect of anti-TGF-β antibodies with P17 in vivo, both molecules were effective. Indeed, AH1-immunized mice remained protected from CT26 tumor challenge if they were treated with anti-TGF-β1 polyclonal antibodies (5 administrations of 100 µg anti-TGF-β1 per mice from day 5 to 9 after AH1 immunization). Similarly, in vivo administration of anti-TGF-β1 antibodies reverted immunotolerance induced by LL-OVA administration (data not show). Although both molecules are efficient TGF-β inhibitors, the use of peptides might have advantages. Thus, the relative short life of peptides would allow a finer control on the inhibition of TGF-β during the required period in vivo. This would reduce the potential toxic effects of long term inhibition of the cytokine.

Tumors produce factors such as prostaglandins, IL-10, VEGF and TGF-β which may create an immunosuppressive microenvironment and may hamper immunotherapy. This microenvironment, and in particular TGF-β1, might favor Treg development. Indeed, it has been widely described that during tumor progression in humans, Treg accumulate in tumors and secondary lymphoid organs (6-12). This increase in Treg number may be favored by a recruitment of naturally occurring Treg cells, as well as by a conversion of CD4+ effector Th cells into Treg, in this particular TGF-β-enriched tumor microenvironment (67, 68). In addition, it has been recently described that the TGF-β2 isoform may induce Foxp3 expression in pancreatic carcinoma cells, enabling these tumor cells to suppress T cell proliferation (49). Thus, inhibition of TGF-β1 and TGF-β2 isoforms would have an impact in this adverse environment by reducing the number of Treg, Foxp3 expression and favoring effector T cell proliferation and function. We show in this work that P17 is able to inhibit the immunosuppressive activity of TGF-β1 as well as TGF-β2 in vitro. P17 administration, after adoptive transfer of CFSE-labeled
CD4⁺ T cells from OT-II transgenic mice to C57BL/6 mice bearing EG.7-OVA tumors, improved proliferation of transferred T cells and reduced the numbers of CD4⁺Foxp3⁺ T cells. Moreover, P17 treatment after the adoptive transfer of CD4⁺ T cells inhibited tumor growth, suggesting that this TGF-β inhibitor might be very useful for the development of anti-cancer therapies.

TGF-β plays a central role in oral tolerance. This takes place via regulation of mucosal inflammation and mediating active suppression against orally administered antigens, (reviewed in (69)). Thus, TGF-β knockout mice develop chronic inflammation in many tissues, including the gastrointestinal tract (70). In addition, recent findings suggest that TGF-β may be a primary link between distinct populations of Treg cells that are induced by feeding. We have recently shown that mucosal delivery of OVA by genetically modified L.lactis (LL-OVA) induces OVA-specific CD4⁺CD25⁻ Treg cells which in turn, suppress OVA specific T-cell responses in DO11.10 mice, in a process critically dependent on TGF-β (33). We have found that P17 administration is able to inhibit induction of oral tolerance in this model, suggesting that P17 may have important applications to enhance the immunogenicity of orally administered antigens.

In summary, we have shown that inhibition of TGF-β by P17 is able to inhibit the immunosuppressive activity of murine and human-derived Treg cells in vitro. Also, and most importantly, in vivo experiments using P17 show that this peptide fosters the immunogenicity of peptide vaccination when administrated 5 days after vaccination. Moreover, P17 was able to improve proliferation of adoptively transferred T cells, and reduce the number of CD4⁺Foxp3⁺ T cells in vivo in mice bearing a TGF-β producing tumor. In addition, P17 was also able to inhibit Treg function in an antigen-specific model of oral induced tolerance. In summary, our results demonstrate that inhibition of TGF-β1 and TGF-β2 with a small synthetic peptide can be a useful therapeutic strategy to enhance the immunogenicity of vaccines or to break tolerance against pathogens or tumor antigens.

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References


Induction of antigen-specific tolerance by oral administration of *Lactococcus lactis* delivered immunodominant DQ8-specific Gliadin peptides in sensitized NOD AB<sup>o</sup> 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\(^o\) 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\(^o\) 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-β and associated with a significant induction of *Foxp3*+ 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-γ 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\(^1\). 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\(^2\) and better therapeutic options are needed\(^3\).

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
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 ABo DQ8 MHC class II transgenic mice. NOD ABo 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 ABo 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.
Methods

Bacteria and media

The genetically modified *Lactococcus lactis* MG1363 (LL) strain was used throughout this study\(^\text{14}\). 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\(^\text{15}\). 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 \(^\text{16}\) (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’ggtgctccagttccataccgaaggcttgc3’ and 5’cgactagttaagcttgtgggttttcttgtgat3’. The amplified fragment was fused to the Usp45 secretion signal of the erythromycin resistant pT1NX vector, downstream of the lactococcal P1 promotor\(^\text{17, 18}\). 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 QGSFQPSQ, 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^o DQ8^+) were backcrossed to NOD mice for 10 generations and intercrossed to produce congenic NOD AB^o DQ8^+ mice, as described previously. NOD AB^o DQ8 transgenic and NOD AB^o 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 QYPSEGSFQPSQENQPA) 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^o 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 ABo 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 \textit{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 \textit{L. lactis}-derived eDQ8d is fully bioactive and that the addition of the E-tag does not interfere with its functionality.

\textbf{Suppression of the DQ8d-induced DTH and peripheral immune response by mucosal delivery of eDQ8d epitopes by \textit{L. lactis} (Figure 3)}

We subsequently investigated the effect of oral administration of LL-eDQ8d on the eDQ8d-induced DTH\textsuperscript{22}. eDQ8d-immunized NOD AB\textsuperscript{o} DQ8 transgenic mice were fed BM9 (inoculation buffer, as a negative
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Figure 3 Mucosal delivery of eDQ8d epitopes by \textit{L. lactis} significantly decreases the DQ8d-induced DTH response and proliferative capacity of bulk spleen and inguinal lymph node cells.

NOD AB\textsuperscript{o} 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 \textit{ex vivo} restimulated with 50 µgr 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.

color), 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\textsuperscript{-2} mm vs 5.1x10\textsuperscript{-2} mm, p=0.0031) (Fig. 3a). Ear swelling was also slightly reduced in LL-pT1NX-treated mice compared to controls (9.3x10\textsuperscript{-2} mm vs 13.1x10\textsuperscript{-2} mm p=0.0343) but to a much lesser degree than in LL-eDQ8d treated mice. NOD AB\textsuperscript{o} mice (without DQ8 transgene) showed
only a minor increase in ear thickness (3.2×10^{-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 (13.1×10^3 vs 5.6×10^3 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 (1.4×10^3 vs 2.8×10^3 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.
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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

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 $5 \times 10^5$ 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 (4.9x10³ vs 1.6x10³ 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).


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**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 (Braat et al submitted and ). 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\(^2^9, 3^0\). 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\(^5, 3^1, 3^2\). Furthermore, recently a separate category of Treg has been described that acquires *Foxp3* upon TGF-β stimulation. These so-called inducible Treg (iTreg) have regulatory functions both *in vitro* and *in vivo*\(^3^3, 3^4\). 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-β/IL-10.

To map the Treg that mediated oral tolerance in our experiments, we studied the functional importance of TGF-β, IL-10, and LAP (membrane-associated TGF-β) on the eDQ8d-specific splenic proliferative response using neutralizing antibodies. Interestingly only the combined neutralization of IL-10 and TGF-β 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\(^3^5-3^7\).

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-β producing Th3 cells drive the differentiation of antigen-specific *Foxp3*\(^+\) regulatory cells in the periphery\(^3^8\). Furthermore TGF-β-dependent conversion of peripheral CD4\(^+\)CD25\(^-\) T cells into CD25\(^+\), CD45RB\(^-/low\) suppressor cells has been reported\(^3^3\). 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\(^low\) lineage\(^3^9\). 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+ Treg40. 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 antigen7, 41, 42. 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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Mouse-strain specific immunogenicity of \textit{Lactococcus lactis}: implications for the use of \textit{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).
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
Th1 adjuvant effect 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 1h 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, Phamringen, 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 (Analis, 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,
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+1 µg 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.
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 2x10^9 to 2x10^7 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+1 µg 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+1 µg 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

**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 \textit{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 \textit{L. lactis} treatment induces \textit{L. lactis}-specific serum IgG2a and IgA antibodies in Balb/c mice**

We further explored whether \textit{L. lactis} treatment induces a \textit{L. lactis}-specific antibody response. A low \textit{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 \textit{L. lactis} specific IgG1 antibodies could be detected, demonstrating that a humoral Th1 response is induced by lactococcus components. Furthermore, also low \textit{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
Th1 adjuvant effect

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

*Lactococcus 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 form 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

**Figure 9.** In BL/6 mice, oral feeding with LL-OVA during feeding regime 2 did not effect the proliferative response of bulk PLN/ILN lymphocytes, but significantly reduced the amount IL-6 and IFN-γ produced by these cells. BL/6 mice were fed LL-OVA or LL-pTREX during feeding regime 2. Non-fed mice were used as control. After feeding, 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, the PLN/ILN lymph nodes were collected and restimulated *in vitro*. Proliferative response and cytokine production were analysed after 90 h and 72 h after *in vitro* restimulation, respectively.
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.

**Acknowledgements**

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Recent advances in clinical practice
Immunopathogenesis of IBD: insufficient suppressor function in the gut?

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Chapter 7

Summary

Activation of mucosal T cells occurs in inflammatory bowel disease and is inhibited by various subsets of “regulatory” T cells, which can be functionally and phenotypically characterized. Regulatory T cells can be generated within the thymus or in peripheral tissues.

In mice, a loss of regulatory T cell activity results in inflammatory bowel disease. Regulatory T cell activity has not been thoroughly studied in inflammatory bowel disease, but currently available data do not provide evidence for a loss of regulatory T cell activity in human inflammatory bowel disease.

Regulatory T cells can be generated or activated in vivo and in vitro using a variety of approaches, including specific small molecules, cytokine-mediated activation, and gene therapy. These cells have therapeutic activity in the preclinical setting.

Immune activation and suppression in inflammatory bowel diseases

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 (1). The causes for this inappropriate response can be attributed to (a combination of) defects in the epithelial barrier, the innate immune response or the adaptive immune response.

Animal experiments as well as clinical data indicate that the immunopathogenesis of Crohn’s disease (CD) and ulcerative colitis (UC) differ at the level of T cell differentiation and activation, although the governing mechanisms responsible for these differences have been incompletely defined. In both diseases, activation of T cells is evident, but pathogenic T cells in Crohn’s disease predominantly produce IFNγ, (membrane bound) TNFα and IL-23, whereas ulcerative colitis is characterized by production of IL-5 and IL-13 (2;3).

The increased production of “Th1” type cytokines in Crohn’s disease is likely related to increased activation of mucosal dendritic cells (DC) and macrophages, and the pivotal function of membrane-associated (Toll like receptor -TLR-) and intracellular (NOD family) receptors in the activation of
these antigen-presenting cells (APC) has now been well established. Both receptors are key mediators of innate host defence, crucially involved in maintaining intestinal homeostasis (4). In healthy subjects, the colonic mucosa harbors ‘non-inflammatory’ dendritic cells, expressing low levels of TLR2 and 4 and producing cytokines such as IL-10, contributing to a non-inflammatory environment(5;6), but in the mucosa of CD patients the production of IL-12 is greatly increased (7-9). DC in both UC and CD have an activated phenotype with higher levels of the activation markers CD40, CD86 and produce more IL-12 and IL-6 compared to controls (5;10).

The causes for this excessive activation are presumably diverse and have been incompletely defined. A minority of CD patients has inactivating mutations within the susceptibility gene NOD2. The NOD2 protein is normally stimulated by its natural ligand MDP, a degradation product of bacterial peptidoglycan (PGN) (11-13). Some studies have shown increased activation of NFκB, which in antigen presenting cells causes increased transcription of IL-12. Indeed, dendritic cells from CD patients with NOD2 mutations produce increased amounts of IL-12 after stimulation with PGN, most likely via loss of inhibition of the simultaneously activated TLR2 pathway (Zelinkova et al. submitted). It should be noted that other studies have shown impaired activation of NFκB in CD patients with NOD2 mutations, suggesting decreased activation (14;15). Hence, it remains unclear what mechanism is responsible for the excessive Th1 profile in Crohn’s disease and whether the underlying genetic defects lead to initial decreased immune activation with failure to clear pathogens, or whether these mutations directly increase activation of immune cells such as DC. Abnormal activation and expression of TLR receptors may also be linked to inflammatory bowel disease: Associations with TLR4 and TLR5 signalling with their bacterial ligands LPS and flagellin, respectively have been reported (16;17), enhanced expression of TLR2 and TLR4 on DCs was observed (5) and recent studies suggest that CD is also associated with TLR9 promoter polymorphisms (18).

Additionally, defective apoptosis of T cells has been suggested to play a role in the pathogenesis and chronic state of inflammation in CD. Lamina Propria T cells from CD patients were shown to be resistant to activation induced cell death (AICD), whereas LP T cells from healthy controls readily underwent apoptosis (19;20). The latter would clarify the effective therapeutic action of anti-TNFα agents such as Infliximab in CD, as this reagent was shown to induce apoptosis through binding to membrane-bound TNF-α (21).

Although the precise cellular and molecular pathways involved remain to be elucidated, these findings give solid and abundant evidence for increased stimulation or dysregulation of the innate immune system.
in inflammatory bowel disease that -in the case of CD- results in the induction of hyperactive T cells, which is probably necessary for the initiation of chronic mucosal inflammation. Increased activation of the innate and adaptive mucosal immune systems is tightly controlled by various regulatory circuits, and it is possible that defects in such mechanisms that normally downregulate intestinal inflammation are insufficient in inflammatory bowel diseases. 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. We also consider the potential therapeutic application of regulatory T cells in IBD.

**Regulatory mechanisms in the gut**

The immune system controls activation of the innate as well as the adaptive arms, through various means primarily including induction of anergy, apoptosis of activated immune cell, and activities of regulatory CD4+ T cells. In addition, several other regulatory mechanisms are operational in the gut mucosa, which are CD8 T cells, γδ T cells and NKT cells, that are highly correlated with their surrounding epithelial cells, and IL-10 secreting B cells, immature DC and plasmacytoid DC. Intra epithelial CD8 T lymphocytes, γδ T cells and NKT cells are mucosal T cell subsets with a restricted T cell receptor repertoire that are in close contact with mucosal epithelial cells (EC). There is evidence that these interactions can lead to the induction of T cells with regulatory capacities: For example, interactions between human intestinal EC and peripheral blood T-cells cause expansion of CD8+CD28− T cells with regulatory activity (22). These cells are present in the LP of healthy individuals, but not in the LP of patients with IBD (23), suggesting that these cells prevent the pathogenesis of IBD. Intra-epithelial lymphocytes have been reported to down-regulate excessive inflammation caused by infection and autoimmunity in epithelial tissues and their protective ability has been shown in several murine models of colitis (24-28).

Accumulating evidence implicates additional cell types in immunoregulation. Recent studies have revealed a protective role of IL-10 producing B cells in murine CD4+ T cell colitis (29) and an inhibition of Ag-specific T-cell proliferation by plasmacytoid DC. This DC population has significant presence in mucosal tissue and is, like steady state LP immature DCs, able to induce a non-anergic state of T-cell unresponsiveness that involves the differentiation of regulatory T-cells (30;31).
These data indicate that mucosal immune activation is regulated at various levels by different cells that down-regulate immune responses. A rapidly expanding body of evidence indicates that the most important among these regulatory cells reside within the CD4\(^+\) T-cell population, and these will be the further focus of this review.

**Regulatory CD4\(^+\) T cells: Phenotype, function and regulation**

Once T cells are activated through engagement of the T cell receptor, they do not distinguish between “self” and “non-self”. It is now clear that the human immune system regards antigens expressed by the normal gut flora, as “self”. Because activated T cells that recognize self-antigens induce significant tissue damage, it is important to either prevent their activation, or control proliferation. It is long known that most high-
affinity self-reactive T cells are clonally deleted within the thymus, but this system is leaky and by itself insufficient to prevent autoreactivity. Hence, prevention of autoreactivity is also continuously controlled outside the thymus, and this “peripheral tolerance” is critically dependent on the presence of regulatory T cells.

Although, in general, microbes mount strong immune responses, the resident gut flora is unable to activate T cells in healthy individuals. Thus there should be a mechanism by which potentially detrimental immune responses in the gut are prevented.

Regulatory CD4⁺ T-cells represent a population of lymphocytes with the ability to suppress both adaptive and innate immune responses (fig 1) (32-34), and these characteristic make them important for both maintenance of immunological tolerance and control of anti-microbial responses. Various types of regulatory T cells have been identified (table 1), but because specific phenotypic markers have long lacked, it is uncertain to what extent these Treg constitute separate lineages. Nonetheless, regulatory T-cells can be divided into two major groups, the so-called ‘naturally occurring’ regulatory T-cells and ‘adaptive’ regulatory T-cells, containing the so-called Tr1 and Th3 cells (35).

<table>
<thead>
<tr>
<th>Feature</th>
<th>Naturally Treg</th>
<th>Adaptive Treg</th>
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<tbody>
<tr>
<td><strong>Subpopulations</strong></td>
<td>CD4+CD25⁺</td>
<td>Tr1</td>
</tr>
<tr>
<td><strong>Site of induction</strong></td>
<td>Thymus</td>
<td>Periphery</td>
</tr>
<tr>
<td><strong>Mechanism of action</strong></td>
<td>Cell-cell contact, cytokine independent</td>
<td>Cytokine dependent</td>
</tr>
<tr>
<td><strong>Characterization</strong></td>
<td>CD25⁺ and Foxp3⁺</td>
<td>IL-10</td>
</tr>
<tr>
<td><strong>Specificity</strong></td>
<td>Self-antigens in the thymus</td>
<td>Tissue specific antigens and foreign antigens</td>
</tr>
<tr>
<td><strong>Protection demonstrated</strong></td>
<td>Transfer colitis SCID</td>
<td>Transfer colitis SCID</td>
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**‘Naturally occurring’ regulatory T-cells**

Most CD4⁺ T cells that recognise auto-antigens in the thymus with high affinity are either clonally deleted, or differentiate into a ‘naturally occurring’ regulatory T cell (Treg). This cell is characterised by a unique phenotype and potent suppressive function towards auto-reactive peripheral T cells. Thymus-derived Treg constitute about 5-10% of mouse and 1-2 % of human peripheral CD4⁺ T cells. Initially, these cells were identified by their CD4⁺CD25⁹ phenotype, but an increasing number
of markers has been recently reported (table 2). Several membrane-expressed molecular markers such as CD25 (IL-2 receptor α chain), glucocorticoid-induced TNFR family-related protein (GITR) and cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) are constitutively expressed on Treg, but can also be observed on activated non-regulatory T cells, and it was not until the discovery of the *foxp3* gene (*FOXP3* in humans) that a unique marker for murine Treg was identified. Mutations in *foxp3* result in severe autoimmune reactivity in both mice and humans, leading to respectively the scurfy or IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked) syndrome. The *foxp3* gene was identified as a master regulatory gene; it is constitutively and specifically expressed in natural Treg and plays an indispensable role in their development and function. Furthermore, forced expression of *foxp3* can convert naïve peripheral blood T cells to Treg cells (36). The specificity of *foxp3* in mice is clear; it is solely expressed in regulatory T cells and the scurfy mutation is always related to defective suppressive function. Conversely, the expression of *FOXP3* in humans is not restricted to Treg and can be induced upon activation of conventional T cells, albeit at much lower levels than in natural Treg. To add to the confusion, it has been reported that IPEX patients have varying degrees of disease severity and not all patients have dysfunctional Treg (37). Even with these restrictions, there is general consensus that *FOXP3* expression is highly correlated to the suppressive function of CD4+ CD25<sup>high</sup> T cells.

It has recently become apparent that expression of the α-chain of the IL-7 receptor, CD127, allows an unambiguous flow cytometry-based distinction of regulatory T cells (CD127<sup>low</sup>) Treg cells and non-regulatory T cells (CD127<sup>high</sup>) within the CD4<sup>+</sup>CD25<sup>+</sup> populations. CD127<sup>low</sup> cells were strongly suppressive in functional suppressor assays and expression of *FOXP3* protein was highly correlated to a CD127<sup>low</sup> phenotype (38;39). These findings are important, because human regulatory T cells can now be accurately identified and isolated.

### Table 2: Cell surface and intracellular markers constitutively expressed by thymus derived natural Treg.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cell surface</th>
<th>Intracellular</th>
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<tbody>
<tr>
<td><strong>Murine</strong></td>
<td>CD25+, CD122+, CD69+, CD44+, CD45RB&lt;sub&gt;low&lt;/sub&gt;, GITR+, CD103+(αE-integrin), CD134+(OX-40), CD54+(ICAM&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>CTLA-4+, Foxp3&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Human</strong></td>
<td>CD25&lt;sub&gt;high&lt;/sub&gt;, CD122+, HLA-DR+(50%), CD45RO+(80%), CD95&lt;sub&gt;high&lt;/sub&gt;, CD45RB&lt;sub&gt;low&lt;/sub&gt;, CD38&lt;sub&gt;low&lt;/sub&gt;, partly CD62&lt;sub&gt;low&lt;/sub&gt;, GITR+, CD127&lt;sub&gt;low&lt;/sub&gt;</td>
<td>CTLA-4+, FOXP3&lt;sup&gt;+&lt;/sup&gt;</td>
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</tbody>
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Chapter 7

Treg were originally thought to be anergic when stimulated \textit{ex vivo}, yet adoptive transfer studies using (DO11.10) TCR transgenic CD4\(^+\)CD25\(^+\) cells have clearly demonstrated the ability of these cells to expand \textit{in vivo} upon T cell receptor stimulation (40). Recently, it has been shown that human Treg can be greatly expanded \textit{ex vivo} by TCR stimulation in the presence of high concentrations of IL-2, since CD25 is functionally essential as a key component of the high affinity IL-2 receptor (41;42), largely increasing their potential for therapeutic manipulation. The exact mechanism of suppression by regulatory T cells remains uncertain. \textit{In vitro}, the suppressive function can be assessed by co-culturing Treg with conventional CD4 (or CD8) T cells (the ‘responder’ T cells) in a Mixed Leucocyte Reaction (MLR). The proliferation of conventional T cells in such assays is induced via TCR-stimulation by allogeneic PBMCs or agonistic anti-CD3 antibodies. In the presence of regulatory T cells, in ratios below to 1/10, the proliferation of the responder T cells and their cytokine production is strongly suppressed. In these \textit{in vitro} studies the suppressive function is cell-contact dependent and independent of cytokines. However, mouse studies have proven the suppression to be dependent on cytokines like TGFβ and IL-10. The mechanisms responsible for these differences between \textit{in vivo} and \textit{in vitro} results remain to be fully explained.

The regulatory T cell executes suppressive function as soon as it is activated via its T cell receptor, either aspecifically (CD28, CD3), by its natural (HLA class II presented) ligand, or by foreign antigens that are cross-reactive to self-antigen receptors (43) in the periphery. Treg not only suppress proliferation, but also downregulate activation, differentiation and even effector function of multiple immune cells including CD4\(^+\) and CD8\(^+\) T cells, natural killer cells, and dendritic cells(44-46). APC are able to regulate Treg activation by differential expressed costimulatory molecules and MHCII molecules. Although the precise mechanism of CTLA-4 expression and its involvement on Treg is not known (47), it is thought that engagement of CTLA-4 by CD80/CD86 on DC activates Tregs, whereas interaction with CD28, in the context of TCR activation, downregulates suppression. In addition, activation of GITR by GITR-ligand (which is expressed on DC) downregulates Treg function (48). An integrin that is expressed by DC, CD103, is also involved in T-cell polarization, promoting a positive balance of regulatory T cell over effector T cell activity in the intestine (49). Interestingly, the very same stimuli (i.e. LPS) that cause APC such as DC to become activated and present antigen, have a direct effect on Treg. Murine CD4\(^+\)CD25\(^+\) Treg express Toll-like receptors 1, 2 and 4-8, and activation of TLR-4 and TLR-5 by LPS and flagellin, respectively activates Treg and increases suppressor
function *in vitro* (50-52). Therefore, TLR activation of Treg seems to counteract uncontrolled activation of T cell proliferation. Conversely, natural ligands for TLR8 and TLR2 can reverse Treg function (53;54). The functional importance of Tregs is underscored by many observations in mice, where depletion of the CD4+CD25+ population precipitates diseases characterized by autoreactive T cells (55-59).

**‘adaptive’ regulatory T-cells**

Apart from the CD4+CD25+ thymus-derived Treg, there is evidence for the existence of Treg that are induced in the periphery, the so-called ‘adaptive’ regulatory T cells. In mice and humans, peripheral conventional CD4+ T cells were shown to differentiate into CD4+CD25+ Treg under the influence of TGF-β in addition to TCR mediated signals (60-62). Alternatively, adaptive Treg which are phenotypically distinct from Treg from intrathymic origin have been identified, and known as the Th3 and Tr1 T cell subsets. These generally do not express CD25 or *foxp3* and are characterised by the secretion of the immunosuppressive cytokines TGF-β and IL-10, respectively. Although their functions are complex and incompletely understood, it seems that their suppressive activity is critically dependent on the production of regulatory cytokines.

A classical example of a peripheral regulatory cells is the Th3 Treg that secretes predominantly TGF-β together with varying amounts of IL-4 and IL-10, and mediates oral tolerance(63;64). The main immuno-suppressive effect of TGF-β is the inhibition of Th1 responses *via* down-regulation of IL-12β2 chain expression, and TGF-β itself is required for differentiation of TGF-β producing cells. Th3-like cells have been shown to be important in some cases of allergy and in autoimmune diseases (65;66).

Tr1 cells were initially isolated from human SCID chimera and subsequently derived by culturing naïve T cells in the presence of high concentrations of IL-10. They secrete high levels of IL-10, a cytokine that inhibits Th1 induction by down-regulation of IL-12 and suppresses the production of pro-inflammatory effector cytokines. Tr-1 cells are anergic, functionally suppressive in vitro, generally produce low levels of TGF-β and IL-5 but no IL-4, and are critically dependent on IL-10 for their function and development (67). In SCID patients transplanted with HLA mismatched haematopoietic stem cells the number of Tr1 cells correlated with tolerance of the host to the graft (37).

The proliferation of murine Tr1 cells *in vivo* is induced by plasmacytoid DC that express low numbers of CD11c and costimulatory molecules, and secrete large amounts of IL-10 (68). Human Tr1 cells can be induced ex vivo with the pharmacological immunosuppressant vitamin D3 and
dexamethasone (69) and by immature (CD83-) DC (70). In contrast to the latter observations, we have demonstrated that induction of Treg that result from activation of monocyte-derived CD11c+ DC by probiotic bacteria requires full maturation of the DC (Braat H et al. submitted). Induction of these Treg is dependent on production of IL-10 by the mature DC, and although these Treg also secrete IL-10, this is not required for their regulatory function.

In summary, peripheral regulatory T cells comprise a heterogeneous group of T cells that secrete immunomodulatory cytokines that have been implemented in various inflammatory conditions.

**Regulatory T-cells in experimental colitis**

It is well known that adoptive transfer of T cells depleted of CD4+CD25+ cells in immunodeficient mice causes multiorgan autoimmunity in the recipient animals (71) and many studies have demonstrated that depletion of CD4+CD25+ T cells in mice aggravates T cell-mediated models of inflammation (72), including colitis (73). Conversely, Treg clearly have anti-inflammatory effects in various murine models of IBD (fig 2). For example, the induction of colitis that results from transfer of CD4+CD45RB\textsuperscript{high} T-cells into immunodeficient mice can be prevented by co-transfer of the antigen-experienced CD4+CD45RB\textsuperscript{low} T-cells. Thymus-derived Treg are CD45RB\textsuperscript{low}, and it is now thought that the CD4+CD25+ Treg present in the CD4+CD45RB\textsuperscript{low} subset are responsible for this regulatory activity (74;75). Co-transfer with isolated CD4+CD25+ T-cells prevents the induction of colitis, which is reverted by the addition of monoclonal anti-CTLA-4, -IL-10R or -TGF-β antibodies. Not only do CD4+CD25+ T-cells prevent the induction of colitis, they also can reverse established colitis and wasting disease, indicating their importance in controlling ongoing immune-mediated inflammation (76).

Peripheral Treg with a Tr1 phenotype also have the capacity to control colitis. Chronic activation of OVA-specific naïve CD4+ T cells in the presence of IL-10 induced Tr1 cells that produced large amounts of IL-10 after exposure to OVA. These cells were able to control colitis induced by pathogenic CD4+CD45RB\textsuperscript{high} T cells in immunodeficient mice, and this function was dependent on activation of the T cell receptor by OVA (77;78). Mouse strains deficient in IL-10 spontaneously develop chronic enterocolitis, underlining the importance of IL-10 in controlling responses against the commensal flora (79-81). Treatment with recombinant IL-10 in the T-cell transfer model prevents but does not cure established colitis. In contrast, local mucosal delivery of recombinant IL-10 by the
The role of regulatory T cells in inflammatory bowel diseases

Because it has long been difficult to reliably characterize regulatory T cells, all data about their presence and functional characteristics in humans are of recent date, and some of these data require confirmation. Several clinical observations indicate that the CD4+CD25+ population in patients with “autoimmune” diseases such as multiple sclerosis, uveitis and autoimmune polyglandular syndromes (APS)-II is functionally defective (87). In patients with Crohn’s disease, about 6% of both peripheral blood and lamina propria (LP) T cells were found to be CD4+CD25+, and the fraction with a high expression of CD25 (CD25bright), expressed CTLA-4 and GITR. In contrast to peripheral blood T cells, some expression of CTLA-4 and GITR was found on lamina propria CD4+CD25− T cells. In concordance with this finding, Foxp3 was predominantly transcribed by CD4+CD25bright LP T cells, and to a lesser extent by CD4+CD25− T cells. When tested for functional properties, it was found that LP CD4+CD25bright T cells, but not CD4+CD25− T cells did suppress the proliferation (as well as cytokine production) of peripheral blood CD4+CD25− T cells (88;89) but not of LP CD4+CD25− T cells. The inability of LP regulatory T cells to suppress proliferation of LP T cells may be related to the relative anergic and memory phenotype of the latter (85). Likewise, CD4+CD25+ T cells isolated from human colonic MLN in UC display typical features of Treg cells and possess potent suppressor activity in vitro in spite of persistent mucosal inflammation {unpublished results, Saruta M et al, DDW 2006 abstract 599}.

At present, these sparse data on regulatory T cells in IBD suggest that the inflammatory pathology in CD patients does not result from an absence or altered functionality of the regulatory T cell population, although the genetically modified bacteria of the strain *L. lactis* seemed to be effective when disease activity was well established, and ameliorated DSS-induced colitis and colitis in IL-10-deficient mice (82). TGF-β is also recognized for its ability to downregulate immune responses. TGF-β1-deficient mice develop a systemic inflammatory response and blockade of TGF-β signalling in T-cells results in T-cell activation and induction of IBD in mice (83). CD4+ Th3 cells protect against uncontrolled inflammation in the gut, and in models of intestinal inflammation TGF-β producing mucosal T cells were shown to reduce disease activity (84-86).
increase in regulatory T cell numbers and activity may be insufficient to suppress the inflammatory condition (90). There are no reliable data on the existence of Tr1 or Th3 cells in the human mucosa, or on their functional properties. Interleukin-10 deficient mice develop inflammatory bowel disease, but patients with IBD do not have deficient IL-10 production (91). Remarkably, isolated T-cells from patients with IBD were found to express high levels of SMAD7, a negative regulator of TGF-β signalling, suggesting that impaired responsiveness to TGF-β may be involved in IBD (92;93). The functional role of LP regulatory T cells may be more complex than that of peripheral regulatory cells, because of the necessity specifically suppress immune responses to endogenous bacteria, but not to bacterial pathogens.

**Therapeutic potential of regulatory T cells**

As described above, Treg can prevent and even cure various experimental colitis models. Although their therapeutic potential is without dispute, translation of these data into therapeutic strategies is not straightforward. Furthermore the ability to apply this therapeutic strategy in a human clinical setting will depend on techniques to isolate and transfer adequate numbers of cells. In most mouse models of autoimmune diseases the antigens that induce T cell activation are known, and antigen-specific regulatory T cells are able to potently suppress activation in an antigen-dependent manner. For example, in the NOD model of autoimmune diabetes, islet antigen-specific BDC2.5 Tregs completely prevent diabetes. However, polyclonal Tregs are at least 50-fold less potent than antigen specific Treg and can only be a viable therapeutic option in this context when sufficient numbers are applied (94).

Although there is evidence for a role of peptidoglycan and flagellin at the level of dendritic cell stimulation, it is unknown what antigens are involved in the pathogenesis of IBD, excluding the possibility to employ antigen-specific Treg. 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 (fig 2). A clear example of bystander suppression was demonstrated in the SCID 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 (95). 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 (96).
Apart from bystander suppression, Treg inhibit the response of conventional CD4 T cells in a contact dependent manner and can even confer suppressive properties to such T cells. This process is known as “infectious tolerance” and results in the conversion of conventional T cells into IL-10 producing Tr1-like cells and TGF-β producing Th3-like cells (97). These concepts of 'infectious tolerance' and bystander suppression are instrumental in providing a context for using Treg as a potential therapy. It has now become possible to produce and expand sufficient CD4+CD25+ and Tr1-like cells for therapeutic application and clinical studies have been initiated. For example in a currently ongoing clinical trial, Roncarolo at al use ex vivo induced Tr1 cells as post-transplant cellular therapy in haematological cancer patients undergoing HLA-haploidentical HSC.

**Figure 2: Bystander suppression** Presentation of bacterial antigens to naive T cells by dendritic cells results in the generation of Th1 effector cells that migrate into the intestine and cause an inflammatory response. Because the antigens that are involved in IBD are unknown, therapeutic application of Treg requires antigen-non-specific suppression. Bystander suppression is the capacity of Treg to suppress immune responses that are caused by a different antigen. The mechanisms involved include the production of regulatory cytokines, deactivation of DC that attempt to stimulate effector T-cells, or direct contact with the responding T-cell. The concept of bystander suppression has been shown for Tr1, CD4+CD25+, Th3 and CD8+ T cells.
transplantation. After a 10 days ex vivo culture of donor PBMC in the presence of irradiated host PBMC and IL-10, the IL-10-anergized donor T cells are infused in the host. The ultimate goal is to provide immune-reconstitution with donor T-cells that are anergic towards host antigens and contain pre-cursors of host specific Tr1-cells. Although promising, the clinical usage of Tr1 cells for the cure of T-cell mediated diseases is still in a developmental stage (77).

A second important observation has been that regulatory functions can be imprinted in mouse and human T cells by genetic engineering. Unselected peripheral blood naïve mouse T cells become regulatory following transfection with a retroviral vector encoding IL-10, and these cells are able to suppress inflammation by a bystander mechanism (98). Such cells can also be generated from human peripheral T cells (99). Using similar techniques, FOXP3 can be overexpressed in human CD4+ T cells but the data on the functional efficacy of these generated suppressor T cells is conflicting(100;101).

Finally, it may be possible to induce regulatory T cells in vivo by directing APC such as DC. It appears that the capacity of DC to induce regulatory T-cells depends on the DC instruction and maturation state (102). Different approaches to render “regulatory” DC(103;104) include ex vivo genetic manipulation, anti-inflammatory cytokine exposure or by direct instruction with tolerogenic compounds. We have recently demonstrated that injection of the Bordetella pertussis-derived filamentous hemagglutinin A (FHA) reduced inflammation in a mouse model of IBD (105). The experimental data of T-cell as well as DC manipulation, along with future investigations needs to determine the exact value of both approaches, but recent advances are very promising.

When considering ex-vivo manipulation or induction of Treg, two major hurdles need to be overcome. Firstly, sufficient numbers of the manipulated T cells need to be directed to the gut mucosa, a process known as “homing”, which is directed by specific integrins and by chemokines. It has been reported that cultured CD4+ gut-derived T cells that express high levels of the pivotal gut-homing receptor α4β7, did not home to the gut following injection in healthy individuals (89). However, we have reported that homing –at least in mice and rats- is greatly increased in inflammatory conditions. Also, it has become apparent that the isolation and expansion of CD45RA+ naïve (instead of CD45RO+) CD4+CD25+ T cells is the best strategy for adoptive Treg cell therapies(106).

Secondly, a problem of adoptively transferred Treg may be that the bystander suppression mechanism is that the targets for downregulation cannot be controlled; this problem needs to be solved. Regulatory T cells
may worsen inflammatory disease, because they may interfere with the immune mechanisms that are necessary for clearance of microbial pathogens (107). The non-specific immunosuppressive effects of Treg are a concern when considering therapeutic application. On the other hand such effects may be limited, because effective pathogen-specific immune responses are shown to be Treg resistant (108). However, from this perspective the use of natural occurring CD4+CD25^{high} Treg may be preferred, as in these cells, at least in vitro, TLR2 triggering results in a temporal loss of the suppressive Treg phenotype (54).

**Conclusions**

Regulatory T cells are key players of immune regulation, and they have important functions in suppressing unwanted inflammatory responses towards self-antigens, and the antigens of endogenous intestinal bacteria (fig 5). IBD patients do not seem to have a primary absolute defect in regulatory cells, but apparently, the regulatory capacity of these cells is insufficient to down-regulate inflammation. None of the current therapies for IBD directly targets Treg function or generation, but drugs that are widely clinically used may influence Treg function. For example, corticosteroids may enhance Treg function in asthma and allergic diseases (109-111) but future research needs to determine the exact role of corticosteroids on regulatory T-cell function. Interestingly it was recently shown that anti-TNF antibodies increased the FOXP3 mRNA and protein levels in the CD4+CD25^{high} compartment and restored their tolerogenic function (112). In mice and humans with diabetes treatment with non-agonistic anti-CD3 antibodies resulted in prevention of progression a loss of islet cell function by immunosuppressive mechanisms that included the induction of Treg (113).

None of the previously discussed possible clinical approaches uses a strategy that allows control over regulatory T cells activity. Are such approaches feasible?

Intravenous administration of relatively large doses of regulatory cytokines is not effective (rhIL-10 administration in IBD patients (114)) or found to be toxic (TGFβ). Only a small fraction of the total administered dose of such cytokines reaches the mucosa and results may be better when such cytokines are locally administered. Mucosal delivery of recombinant IL-10 by genetically modified bacteria such as *L.lactis* addresses this problem and was indeed shown to ameliorate DSS colitis and colitis in IL-10/- mice. Recently, we have demonstrated in a clinical phase 1 trial that this engineered cytokine-excreting organism can be safely administered to
Crohn’s disease patients and is biologically contained (115). TGFβ can also be locally expressed, for example by delivery of TGFβ-encoding plasmids to mucosal surfaces and this approach is in the phase of pre-clinical trials(116).

Contained immunosuppression can also be accomplished by modifying or expanding T cells with regulatory properties \textit{ex vivo}. This strategy involves a harvesting step that yields peripheral blood T lymphocytes (for example by apheresis) followed by forced differentiation by exposure to cytokines and tolerogenic compounds, or by genetic engineering with a “regulatory” gene. Upon readministration to the patient, such cells can down-regulate inflammation by a bystander mechanism, following specific integrin-mediated homing. It is technically feasible to specifically expand T cells with a predefined T cell receptor, that can be specifically activated by an orally administered antigen, allowing for control of the immune suppression. These strategies are attractive in view of the long lifespan of T cells, and are expected to have long-term effects.

Regulatory T cells are extremely potent and production of low amounts of IL-10 by a very small fraction of mucosal T cells (IL-10-engineered T cells that comprised only $\sim 0.001\%$ - of all mucosal T cells were effective) a sufficient therapeutical effect can be achieved (117). With the identification of more genes that determine regulatory T cell development, the ability to identify Treg using cell surface markers and with improving transduction methods over time, the possibilities for such approaches will be significantly expanded.

**Acknowledgments**

We would like to thank Professor Dr. H.Spits for critical reading of the manuscript.

**References**


Chapter 7


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Chapter 7


Teaching Tolerance With a Probiotic Antigen Delivery System

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Chapter 8


The gastrointestinal (GI) tract is constantly exposed to a diverse set of antigens of dietary, microbial, and host origin. These luminal antigens influence the host innate and adaptive immune systems through interactions with intestinal epithelial cells and immune cells residing in the lamina propria. Although these dietary and microbial antigens are “foreign,” the human immune system has developed exquisite regulatory mechanisms to prevent unwanted and potentially pathogenic immune responses to these innocuous molecules—leading to a state of unresponsiveness or “tolerance.” Indeed, a breakdown of tolerance can lead to various disorders, including food allergies and inflammatory bowel diseases (IBD).\(^1\)\(^-\)\(^3\) Recently, investigators have attempted to develop strategies to induce such tolerogenic mechanisms to prevent a hyperactive immune system associated with a variety of diseases.

Induction of suppression of immune responses to an antigen by its prior oral administration is defined as “oral tolerance.”\(^4\) There are 2 main mechanisms by which oral tolerance is achieved: (1) deletion/anergy of pathogenic antigen-specific T-cell clones; and (2) induction of regulatory/suppressor T cells (Tregs), cells with the unique ability to actively control effector immune responses. Although controversial, a contributing factor implicated in determining which mechanism occurs after oral administration of an antigen is antigen dosage. Low doses of antigen favor the development of regulatory T cells, whereas high doses result preferentially in deletion/anergy of reactive T-cell clones.\(^5\)\(^-\)\(^7\) However, it seems likely that both regulatory mechanisms (deletion/anergy of specific T-cell clones and regulatory T-cell induction) may occur simultaneously in vivo. Although deletion/anergy of specific T-cell clones and regulatory T-cell induction require antigen specificity, regulatory T cells stimulated in an antigen-specific manner can actively suppress other T cells in an antigen-independent manner (ie, bystander suppression).\(^8\)

The first description of the induction of a regulatory T-cell population with tolerogenic properties following prior oral feeding of a specific antigen was reported in the experimental autoimmune encephalomyelitis model. These regulatory cells, defined as Th3 cells, secrete transforming growth factor (TGF)-β, possess suppressive properties in vitro, and are capable of transferring tolerance to non-fed animals.\(^9\)\(^-\)\(^11\) Th3 cells are part of a broader family of adaptive regulatory T cells (aTregs) that are peripherally activated, and whose suppressive activity is, at
least in part, cytokine dependent. This family also includes interleukin (IL)-10-secreting cells or Tr1 cells that were originally described as T-cell clones arising in vitro after prolonged stimulation with IL-10. Tr1 cells were capable of protecting against colitis induced by transfer of CD4⁺CD45RB<sup>hi</sup> T cells into lymphopenic hosts. Interestingly, the gut flora, and more specifically Helicobacter hepaticus, seem to modulate the presence of Tr1 cells in the GI tract.

It has become clear over the past few decades that a fraction of peripheral T cells that maintain tolerance to self-antigens exist, in contrast with nTregs, under normal physiologic conditions. These cells are defined as naturally occurring regulatory T cells (nTregs). nTregs are generated in the thymus and express the forkhead-box transcription factor Foxp3, a molecule involved both in nTreg cell development and function. Other associated surface markers expressed by nTregs include the IL-2Ra chain (CD25), GITR, and CTLA-4. nTregs have been shown to play a nonredundant role in preventing auto-immune disease, preventing graft-versus-host disease, suppressing antitumor immunity, and modulating immune responses to infectious pathogens. In mice, transfer of nTreg cell-depleted CD4⁺ cells into lymphopenic hosts leads to severe chronic colitis. Furthermore, genetic targeting of key molecules in nTreg cell function/homeostasis (ie, CD25, IL-2, Foxp3) results in colitis in mice. Thus, nTregs appear to play a central role in regulating gut immune homeostasis; however, the extent by which nTregs contribute to oral tolerance is still unknown. In this regard, Thorstenson et al have reported the appearance of an ovalbumin (OVA)-specific CD4⁺ CD25⁺ cell population after oral administration of low doses of OVA to WT mice adoptively transferred with OVA-specific T cells and have shown that these cells are suppressive ex vivo in a TGF-β- and IL-10-independent manner. Nonetheless, it remains unclear whether these cells are bona fide thymic-derived nTregs.

A third category of regulatory T cells has been described that acquire Foxp3 expression upon TGF-β stimulation. These so-called inducible regulatory T cells (iTregs) may be related to Th 17 cells that arise in vitro in the presence of both IL-6 and TGF-β. Inducible Treg cells have regulatory functions both in vitro and in vivo. Interestingly, maintenance of these cells in culture with TGF-β induces TGF-β secretion. Thus, TGF-β-dependent regulation is central to both Th3 and iTreg development/function and suggests that some overlap may exist between these 2 cell types in vivo.

Although oral tolerance has been effectively achieved in mice using various systems, clinical attempts in humans have thus far been unsuccessful. In this issue of Gastroenterology, Huibregtse et al
developed a novel approach of oral antigen delivery in mice that employs the probiotic bacterial strain *Lactococcus lactis* as an antigen delivery system. This approach has several features that may make it an attractive antigen delivery system for the use in modulating immune responses in humans.

*Lactis* is a Gram-positive, noncolonizing, nonpatho-genic, noninvasive bacterium that is not normally present in the human or mouse gut flora and is commonly used as a fermenting agent in the food industry. Previous work by Steidler et al.\(^2\) showed that a strain of *Lactis* modified to express IL-10 was effective at preventing colitis in IL-10 knockout mice and after oral administration of dextran sodium sulfate. *Lactis* has also been used to stimulate IL-10 secretion in the GI tract when expressing the low-calcium-response V antigen (LcrV) protein from *Yersinia pseudotuberculosis*.\(^2\) Oral administration of LcrV-secreting *Lactis* was effective at protecting mice from experimental colitis.\(^2\) Recently, a
Phase I trial was conducted in humans with moderate to severe active Crohn’s disease that demonstrated that IL-10-secreting *L. lactis* was safe and may be effective for the treatment of Crohn’s disease. Probiotics can be defined as live microorganisms that have a beneficial effect to the health or well-being of the host. *L. lactis* is a member of the lactic acid bacteria family, which are among the most commonly used probiotic agents. These bacteria have the ability to induce Th1-cytokine production by splenocytes in vitro and inhibit Th2 cytokine production by mononuclear cells from allergic patients. Moreover, *L. lactis* has an adjuvant effect when co-administered with antigen in murine models of birch pollen or food allergies. These models are associated with Th2 responses and one of the postulated mechanisms of action for *L. lactis* is the stimulation of counter-regulatory Th1 responses.

In the current report, Huibregtse et al. show decreased delayed-type hypersensitivity in OVA-specific T-cell receptor (TCR)-transgenic mice that were fed *L. lactis* engineered to express OVA (LL-OVA) compared with those fed high- or low-dose soluble OVA. This response was accompanied by an up-regulation of IL-10 in the gut-associated lymphoid tissue (GALT) and spleen of the tolerized animals in an antigen-specific manner. Of note, although this response was more pronounced after LL-OVA administration, mice receiving *L. lactis* alone, without OVA expression, also partially responded. Although IL-10 induction was specifically up-regulated after LL-OVA administration, partial suppression of splenocyte proliferation and interferon-γ secretion was achieved with *L. lactis* alone.

An important advance of this study is assessing the role of the mode of antigen delivery in tolerance induction. The precise mechanism by which *L. lactis* enhances tolerogenic signals remains unclear. *L. lactis* may directly modulate antigen processing, antigen presentation, and/or expression of co-stimulatory molecules on dendritic cells (DCs) through stimulation, at least in part, of innate immune molecules. Most of the luminal OVA found after oral gavage was in the cecum and colon, and most of the mucosal OVA was in the terminal ileum. It is unknown which site is most important for tolerance induction. DCs residing throughout the GI tract have been shown to directly sample luminal antigens through the epithelial cell layer. Through an association with the intestinal epithelium, *L. lactis* may permit more efficient antigen uptake than is available through oral administration of OVA. Alternatively, the authors report mucosal associated OVA in the terminal ileum and cecum in LL-OVA-fed mice. A bacterial-delivered antigen may provide a more effective dose of antigen to the intestine.
than obtained with soluble oral antigen alone. The authors have not evaluated whether similar suppressive effects occur in the presence of \textit{L lactis} plus soluble antigen, which would point to whether bacterial expression of antigen is critical. Furthermore, whether LL-OVA antigen delivery modulates anergy/deletion of a specific T-cell clones was not assessed.

As mentioned previously, TGF-\(\beta\) regulates both the function of Th3 cells and development of iTregs. In this issue of Gastroenterology, Huibregtse et al\textsuperscript{25} demonstrate that oral administration of LL-OVA leads to the appearance of a suppressive T-cell subset whose activity was almost completely dependent on TGF-\(\beta\)—a classic property of Th3 cells. Through adoptive transfer experiments, they further refine the suppressive activity to a CD4\(^+\)CD25\(^-\) T-cell subset—a subset that does not typically include nTregs. Interestingly, these cells expressed Foxp3 and CTLA-4, a phenotype consistent with iTregs.

As noted, IL-10 was up-regulated in the 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. A similar cross-talk between IL-10 and TGF-\(\beta\) has previously been reported in experimental co-litis.\textsuperscript{38} Taken together, these results highlight how different Treg populations can overlap in function and phenotype and hint at the complexity of the regulatory pathways involved in oral tolerance.

Several immune-mediated diseases (such as food allergies) are triggered by well-defined antigens; therefore, tolerance protocols aimed at targeting these antigens are clearly warranted. In the current report, Huibregtse et al\textsuperscript{25} successfully induced antigen-specific immune tolerance in TCR-transgenic mice. Whether antigen-specific immune suppression can be achieved in a normal host bearing a broader TCR repertoire remains unknown. Regardless, nonspecific bystander suppression of immune responses may be beneficial in the context of a disease driven by multiple antigens or where specific antigen triggers have remained elusive (eg, IBD). Oral administration of OVA using \textit{L lactis} may trigger both antigen-specific as well as antigen-nonspecific regulatory pathways.

In conclusion, this is the first report demonstrating that \textit{L lactis} can be used to efficiently deliver antigen to the intestinal mucosa for the induction of antigen-specific peripheral tolerance (Figure 1). 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 \textit{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.

References


Nieuwe strategie voor de behandeling van allergische aandoeningen.

Orale inname van allergenen of auto-antigenen via de melkzuurbacterie (Lactococcus lactis) kan een nieuwe strategie zijn voor de behandeling van allerhande auto-immune en allergische aandoeningen. VIB-onderzoekers verbonden aan de Universiteit Gent toonden - in samenwerking met geneesheren van het AMC (Amsterdam) - aan dat via de melkzuurbacterie auto-antigenen of allergenen oraal toegediend kunnen worden.

ActoGeniX - een spin-off van VIB en Universiteit Gent - ontwikkelt reeds vandaag, op basis van het geoptroooidere technologieplatform, een hele reeks nieuwe biofarmaceutische geneesmiddelen met een brede waaiers van toepassingen.

Het immuunsysteem


Lactococcus als leverancier van medicijnen

In haar natuurlijke vorm is de melkzuurbacterie Lactococcus lactis een bekende voedselbacterie die sinds mensenheugenis gebruikt wordt voor de omzet van melk in kaas en yoghurt. In de strijd tegen chronische darmziekten hebben VIB-onderzoekers L. lactis reeds ingezet als producent van een geneesmiddel tegen darmontstkingen. De eerste klinische testresultaten hiervan zijn veelbelovend.


Getest op muizen

Inge L. Huibregtse (AMC) en Veerle Snoeck (VIB) evalueerden het gebruik van OVA-secreterende bacteriën bij muizen die overgevoelig waren voor ovalbumine. Het toedienen van OVA-secreterende bacteriën, die ovalbumine op de juiste plaats in de darm afleveren, resulteerde in ovalbumine-tolerante muizen.

Veelbelovend

Uit dit onderzoek blijkt dat L. lactis ingezet kan worden om tolerantie t.o.v. bepaalde stoffen

Relevante wetenschappelijke publicatie: Huibregtse et al., Induction of OVA-specific tolerance by oral administration of Lactococcus lactis secreting OVA, Gastroenterology, 2007.

NRC Handelsblad: 020807 voorpagina

Bacterie ingezet tegen allergieën

Allergieën kunnen straks mogelijk beter behandeld worden dankzij de melkzuurbacterie die gewoonlijk wordt gebruikt voor het maken van yoghurt en kaas.

Pg 9

Bacterie ingezet tegen allergieën

Door een onzer redacteuren

Rotterdam, 2 aug. Allergieën kunnen straks mogelijk beter behandeld worden dankzij de melkzuurbacterie die gewoonlijk wordt gebruikt voor het maken van yoghurt en kaas. Het slikken van een genetisch veranderde versie van Lactococcus lactis zorgt dat allergische muizen hun overgevoeligheid kwijtraken.

Archief:

overzicht - Meer wetenschapsnieuws

Dat schrijven onderzoekers van het AMC en de Universiteit Gent in het augustusnummer van tijdschrift Gastroenterology. Allergie wordt veroorzaakt doordat het immuunsysteem overdreven reageert op onschuldige stoffen uit bijvoorbeeld stofmeel (hooikoorts), pinda’s (voedserre) of uitwerpselen van de huisstofmijt. Een systematische, lichte blootstelling aan de stof die de reactie veroorzaakt – het allergen – kan de overgevoeligheid verminderen. Tot nu toe is er echter geen adequate manier om het lichaam met allergenen in contact te brengen.

Daar denken de wetenschappers iets op te hebben gevonden. Zij willen Lactococcus lactis gebruiken om allergenen heel gericht in de darmen te krijgen; een deel van het lichaam dat in het bijzonder geschikt is om tolerantie in het hele lichaam op te wekken. Om deze werkwijze te testen, plaatsten de onderzoekers het gen voor het eiwit ovalbumine in het DNA van de bacterie, waardoor deze ovalbumine ging produceren. De transgene bacteriën voerden zij aan muizen die overgevoelig zijn voor het eiwit. Al een uur daarna bleken de bacteriën ovalbumine in het darmstelsel uit te scheiden.

Gewoonlijk krijgen deze muizen zwellingen in de oren wanneer zij worden ingespoten met ovalbumine. De oren van muizen die de transgene bacterie hadden gegeten, zwollen echter 15 keer minder.

Uit eerder onderzoek was al gebleken dat het niet werkt om het allergeen zelf te eten. Waarschijnlijk wordt deze grotendeels afgebroken in de maag. Ook in dit onderzoek bleken de oren van muizen die verschillende doses ovalbumine hadden gekregen even hard te zwellen als de muizen die een controlostdos hadden gegeten. Overigens zouden voor medicaal gebruik grote hoeveelheden zuiver allergen nodig zijn, wat duur is om te produceren.

Of deze strategie ook bij mensen werkt, is nog de vraag. Het helpt wel dat Lactococcus lactis een onschuldige bacterie is; wie kwark of yoghurt eet krijgt hem immers ook binnen. Eerder werd de bacterie al succesvol gebruikt bij patiënten met de ziekte van Crohn. Bij hen werd hij ingezet om de ontstekingsremmer interleukine-10 af te leveren in het ontstoken maagdarmkanaal.
Telegaaf: 020807 pg 9:

Aangepaste bacterie pakt allergieën aan

AMSTERDAM, donderdag

Belgische wetenschappers hebben samen met artsen van het AMC in Amsterdam een methode ontwikkeld waarmee men met behulp van bacteriën allergieën geneest.

Hierdoor moeten mensen met zeer ernstige allergieën en auto-immuunziekten weer een normaal leven kunnen leiden. Het gaat daarbij om onder meer astma, hooikoorts, glutenallergie, de ziekte van Crohn en reumatoïde artritis.

De allergieën en auto-immuunziekten worden veroorzaakt doordat ons systeem, in de dagelijkse strijd tegen schadelijke stoffen en micro-organismen die het lichaam binnen willen dringen, het verschil niet meer herkent tussen lichaamseigen en lichaamsvreemde stoffen. Vandaar dat het systeem de eigen weefsels en organen aanvalt.

In andere gevallen reageert het immuunsysteem verkeerd op onschuldige stoffen als huismijt, melkproducten en stuifmeel. Deze overdreven reactie staat bekend als een allergie. Volgens de Belgische onderzoeker dr. Pieter Rottiers lijdt 20 procent van de Europese bevolking aan een of andere allergie: "Dit is een verdubbeling ten opzichte van 15 jaar geleden."

Aanval

De Belgische onderzoekers slaagden erin een melkzuurbacterie zodanig genetisch te wijzigen dat het immuunsysteem wordt hergeprogrammeerd, waardoor het niet meer de aanval opent op pollen, melk of gluten.

Op een congres kwamen de Belgen in aanraking met Nederlandse artsen van het AMC Amsterdam. Daarop werd besloten een proef in Nederland te houden met tien patiënten met de ziekte van Crohn. "De eerste resultaten waren positief. Zo waren er geen toxicologische bijwerkingen. We willen nu op grotere schaal een proef gaan doen met 30 patiënten." Volgens dr. Rottiers gaat het vijf tot tien jaar duren voor de medicijnen beschikbaar zijn.
Melkzuur kan helpen bij genezen van allergie

Allergieën kunnen mogelijk genezen worden met behulp van genetisch gemanipuleerde melkzuurbacteriën (*Lactococcus lactis*). Door deze bacteriën zo te veranderen dat zij het eiwit uitscheiden waar tegen de allergie bestaat (aller geen), kan het afweersysteem in de darmen geleidelijk worden blootgesteld aan het allergene. Dit kan mogelijk leiden tot tolerantie voor het allergene. In het vakblad *Gastroenterology* beschrijft I. Hui jibregtse (AMC Amsterdam) dat dit principe werkt bij muizen.

Provinciale Zeeuwse Courant aug 2007
دیانشمند بالینیکی راه حلی برای آثرزی یافتند

دو دیانشمند بالینیکی به همراه هنده خود تنوانستند با استفاده از گونه‌ای باکتری که در شیر یدا می شود رویی برای مبارزه با آثرزی (حساسیت) پیدا کنند.

پیشنهاد کننده ی گرامی

از مطالعه اینترنت و ایده‌هایی که در زمینه ی پریبارتر کردن این سایت سیاست‌گذار خواهیم بود.

برای تمامی یا ما اینجا کلیه کنید

در این کار گروهی از آقای Veerle Snoeck و خانم togemin Ac از آموزش و پرورش و Pieter Rottiers از شرکت viB و خانم Inge Huibregtse از هنده توانستند روشی نوین را پایه‌گذاری کنند.

آنا در پایانگوشه ی این باکتری که در استیتاکتیک وجود دارد می توانند در بنان جانداران به گنگ برخی از مواد (که معمولاً دنیا روی می زند)

کارآمد باند. آنها در آزمایش‌های خود از موس استفاده کرده‌اند.

این دستورالعمل پست از پژوهش‌های پیشی رفت توان در مبارزه با بیماری‌های آثرزی و خود مصنوع کردن (autoimmune) کاربرد داشته باشد.

هرچند گاه‌های زیادی یک‌تا دو پایان راه برداشتند شود ولی برنده این کار با کمک این روش نوین گماشته شده است.

گفتگوی است که بخش ۲۰ درصد از مردم اروپایی بیش از آلتزی ها دست و پنجه نرم می کنند که به نسبت ۱۵ سال گذاشته ۲ برابر شده است.

هم اکثریت بیماری‌های آلزایمر مدای شد و لذا می توان با چک داروها عوارض آنها بهبود بخشید. روشن نهایی‌تر بسیاری برای بیماران دارویی بروی

ریشه چنین کردن آلزایمر می‌باشد.

- در همین زمینه;
- گزارش: گزارش دانش‌مند دست‌مبتنی در بالینیک
- سببکه‌های بهتر برای افراد نشان‌دهنده است
- دیاگنازی آلتزی یا عفونت‌های می‌دهد
- دانشمندان بالینیکی یافتند گیاه‌اتن ساختند
- بالینیکی چه بیماری‌هایی دارند؟
- آلتزی یا حساسیت چیست?
- گیاه‌های ضد حساسیت ساخته شدن
- گزارش: سرم‌مورف‌داری بالینیک ایمیلی شد
- کارآفرینان بالینیکی: ۱۲ روز بیماری در سال
- نگرش بر تدریسی همگانی در بالینیک
- دوپامین بالینیکی: نهایتی نسبتی ی از ارزای بلوسی
- تکه‌های دارودی مورف‌سازگاری
- اهمیت و کاربرد سرم‌مورف‌داری
- کستر بیماری‌های آلزایمر در ایران
- شایعات بالینیک بی‌پاران کمک ممکن
- الکترون بی‌پاران در بالینیک
- جایی در اروپا و بالینیک

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Summary and general discussion

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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 *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 *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<sup>0</sup> DQ8 mice. We furthermore show that this effect is dependent on the secreted antigen but also on yet undefined characteristics of *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. *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 probiotica. Previously, several genetically modified *L. lactis* strains have been generated for local synthesis and delivery of immunomodulatory proteins to the intestinal mucosa. For example a strain of *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 *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 *L. lactis* secreting IL-10 is clinically and biologically safe and consequently oral administration of genetically modified *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 \textit{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 \textit{L. lactis} enhances tolerogenic signals remains unclear, but it is possible that OVA peptide and/or lactococcal antigens and/or whole \textit{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 compromising 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, \textit{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 \textit{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.
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 \textit{in vitro} and \textit{in vivo}.

In this thesis we demonstrate that active in situ synthesis and mucosal delivery of OVA by the genetically engineered \textit{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 (\textbf{Chapter 3}) and that TGF-β neutralizing peptide (p17) completely abolished LL-OVA induced antigen specific oral tolerance \textit{in vivo} in DO11.10 mice (\textbf{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 \textit{in vitro} and \textit{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 AB\(^{\circ}\) 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 AB\(^{\circ}\) 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 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.
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Nederlandse samenvatting

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Chapter 10

Nederlandse samenvatting

Het maag-darmstelsel

Het maag-darmstelsel heeft een zeer belangrijke maar gelijktijdig complexe functie. Enerzijds moeten er zoveel mogelijk voedingsstoffen opgenomen worden, anderzijds moeten schadelijke bacteriën tegengehouden worden. Om onderscheid te kunnen maken tussen schadelijke (ziekteverwekkende) en niet schadelijke bacteriën en voedingsstoffen, is de darm uitgerust met een aantal natuurlijke afweermechanismen. Hieronder vallen de continue darmbekleding met epitheelcellen, de door deze cellen geproduceerde slijmlaag (mucus) en specifieke cellen van het immuun(afweer)systeem. De normale respons van het immuunsysteem op een ziekteverwekkende bacterie is een ontstekingsreactie, de normale respons op niet schadelijke voedingsstoffen en bacteriën is de inductie van orale tolerantie. Dit is een actieve afweeronderdrukkende respons.

De tekstboek definitie van orale tolerantie is een specifieke immunologische onachtzaamheid voor een eiwit challenge geïnduceerd door herkenning van eerdere voeding. Bij een aantal aandoeningen van het maag-darmstelsel is dit discriminerende vermogen gestoord en zou het herstel van orale tolerantie de ideale therapie zijn. Onder deze ziektes vallen coeliakie, IBD en verscheidene allergieën.

Coeliakie

Coeliakie, oftewel glutenallergie is een intolerantie cq onverdraagzaamheid, voor gluten, de in alcohol oplosbare eiwitfractie van tarwe, haver, rogge, gerst, spelt en kamut. Bij mensen met coeliakie veroorzaakt voedsel dat gluten bevat, beschadiging van het slijmvlies van de dunne darm (vlokatrofie), waardoor het normaal zeer grote darmoppervlak gereduceerd wordt tot een zeer klein oppervlak voor voedselopname. Als gevolg hiervan kunnen klachten ontstaan als diarree, verstopping, groeistoornissen, humeurigheid en vermoeidheid. Ook kunnen tekorten ontstaan aan onder meer vitamines en ijzer. Het is een veel voorkomende, maar vaak ongediagnosticeerde aangeboren darmziekte, naar schatting heeft 1 op de 200 mensen in de westerse wereld er last van. Hetgeen betekent dat er in Nederland ongeveer 75.000 patiënten zijn. Van deze groep is er echter maar een gedeelte, ongeveer 10.000 patiënten...
Nederlandse samenvatting

IBD
IBD (Inflammatory Bowel Disease), oftewel de ziekte van Crohn en colitis ulcerosa, is een chronische ontsteking van de darm. Momenteel hebben ongeveer 35.000 mensen in Nederland IBD. Er zijn duidelijke aanwijzingen dat zowel erfelijke factoren als omgevingsfactoren zoals stress en roken een rol spelen bij het ontstaan van deze ziekten. De darmklachten zelf zijn afhankelijk van de ernst van aantasting en de plaats(en) waar de ontstekingsverschijnselen zich voordoen, maar gaan meestal gepaard met diarree, buikpijn en koorts. Bij de ziekte van Crohn kunnen de ontstekingen in het gehele maag-darmstelsel voorkomen, van mond tot anus, maar zijn ze meestal gelokaliseerd in het laatste gedeelte van de dunne darm en de dikke darm. De aandoening manifesteert zich meestal bij jongvolwassenen en de klinische symptomen bestaan hoofdzakelijk uit buikpijn, diarree en gewichtsverlies. De oorzaak van de ziekte is nog niet helemaal bekend, maar duidelijk is dat de ziekte aangeboren is, waarbij verschillende genetische afwijkingen een rol spelen. Het is waarschijnlijk dat de aard van de oorzakelijk genetische afwijkingen niet voor alle patiënten met de ziekte van Crohn identiek is, maar een slecht gecontroleerde immuunrespons in de darm tegen de aldaar aanwezige normale bacteriële flora, die gepaard gaat met ontsteking is altijd het gevolg. De huidige behandeling van de ziekte van Crohn bestaat uit toediening van corticosteroïden en immuunsuppressieve middelen zoals azathioprine en methotrexaat. De laatste jaren is er een belangrijke plaats voor behandeling met antistoffen gericht tegen tumornecrosefactor (infliximab en adalimumab). De geneesmiddelen hebben vooral een gunstige werking bij het bestrijden van actieve ontstekingen, maar een zeer beperkt vermogen om opvlammingen van de ziekte van Crohn te
voorkomen. Daarbij gaan ze vaak gepaard met korte en lange termijns bijwerkingen. Er is daarom behoefte aan nieuwe therapieën die op de lange termijn effectief zijn, recidieven kunnen voorkomen en gepaard gaan met minder bijwerkingen.

Orale tolerantie als therapie

Voor beide aandoeningen zou de inductie van orale tolerantie een ideale therapie zijn in plaats van het vermijden van het uitlokkende antigen (eiwit) of een niet specifieke immuun suppressie. Zoals hierboven beschreven is de tekstboek definitie van orale tolerantie een specifieke immunologische onachtzaamheid voor een eiwit challenge geïnduceerd door herkenning van eerdere voeding. Het is duidelijk dat dit als therapie vele voordelen heeft zoals een antigen-specifieke afweersysteem onderdrukking, het ontbreeken van toxiciteit en de mogelijkheid om het chronisch te geven, mede hierom wordt het al jaren gebruikt als therapeutisch optie. Maar helaas zijn de verschillende methodes van aanpak tot op heden niet succesvol gebleken. Momenteel is het exacte mechanisme van de inductie van antigen specifieke orale tolerantie niet duidelijk. Wel is het duidelijk dat er 2 belangrijke paden cq manieren zijn om het te induceren; de eerste is door toediening van een hele hoge dosis antigen waarna clonale ongevoeligheid of deletie van de cellen volgt en de tweede is door de toediening van hele lage dosis antigen hetgeen de inductie van regulatoire T-cellen (Treg) teweeg brengt. Regulatoire T-cellen zijn cellen met de unieke capaciteit om de immuun response te onderdrukken. Van de laatste strategie hebben wij gebruik gemaakt bij de ontwikkeling van onze nieuwe experimentele therapie.

Genetisch gemedificeerde melkzuurbacteriën

In dit proefschrift beschrijven we een nieuwe experimentele therapie, bestaande uit de genetisch gemedificeerde bacteriën, Lactococcus lactis die in staat zijn om ter plaatse van de darmwand zeer lage dosis antigen te maken en uit te scheiden en zo regulatoire T-cellen te induceren. Lactococcus lactis is een melkzuurbacterie, dit zijn niet ziekteverwekkende, grampositieve bacteriën, die suiker omzetten in melkzuur. Verscheidene soorten en stammen melkzuurbacteriën worden gebruikt voor de conservering van levensmiddelen, zoals kaas, yoghurt,
karnemelk, zuurkool en zuurdesem. Melkzuurbacteriën zijn normale, niet-ziekteverwekkende bewoners van de menselijke darm. Nu hebben we deze bacteriën zo aangepast op DNA niveau om de inductie van antigeen specifieke orale tolerantie (eiwit specifieke afweer onderdrukking) tegen LL afgegeven antigenen (eiwitten) te bewerkstelligen. Vanzelfsprekend opent deze aanpak de weg voor LL-gemedieerde inductie van orale tolerantie als therapie voor ziektes die resulteren van een antigen gedreven afweer reaktie, zoals verscheidene auto-immuun, inflammatoire en allergische aandoeningen.

In hoofdstuk 1 geven we een algemene introductie over de belangrijkste onderwerpen van dit proefschrift. Hieronder vallen de normale fysiologie van het immuunsysteem van de darm en antigen-specifieke immuunresponsen (afweer respons op ‘vreemde’ eiwitten). Verder wordt er achtergrond informatie gegeven over orale tolerantie en de belangrijkste cel types die hierbij een rol spelen.

Hoofdstuk 2 beschrijft een andere strategie voor de inductie van tolerantie middels de Lactococcus lactis die het humane anti-inflammatoire cytokine, interleukine 10 (LL-hIL10) aanmaakt en uitscheidt. Hier kijken we naar het effect van LL-hIL10 op de modulatie van humane dendritische cellen (DC) en de daarop volgende T-cel respons. De door LL-hIL10 geïnduceerde regulatoire T-cellen zijn in staat om bystander T-cellen te onderdrukken alleen als de dendritische cellen volledig gematureerd zijn met behulp van MF (maturatie faktor). Tevens laten we zien dat zowel de LL-hIL10 blootgestelde DC en daarop volgende T-cellen beduidend meer IL-10 produceren, hetgeen een belangrijke rol speelt bij de inductie maar niet bij de functie van de regulatoire T cellen. De klinische effectiviteit van deze bacteriën is al aangetoond in een fase 1 klinische trial in patiënten met de ziekte van Crohn, maar deze data verduidelijken en versterken de relevantie van het mechanisme in vivo.

Hoofdstuk 3 omvat een nieuwe strategie voor de inductie van orale tolerantie door de productie en secretie van lage dosis antigen door de genetisch gemodificeerde Lactococcus lactis. Als modeleiwit hebben we OVA (ovalbumine) gebruikt en gekeken naar de lokale en systemische afweer reacties in OVA T-cel receptor transgene muizen (DO11.10) na orale toediening van deze genetisch gemodificeerde Lactococcus lactis OVA (LL-OVA). De resultaten tonen aan dat de LL-OVA in staat is om het eiwit (OVA) in de mucosa (darmwand) af te leveren en dat dit onderdrukkend van de lokale en systemische afweer respons geeft. Tevens laten we zien dat achterliggende mechanisme de inductie van CD4+CD25- regulatoire
T-cellen is, die functioneren door een TGF-β afhankelijk mechanisme. Met andere woorden laten we zien dat de mucosale antigen afgifte door genetisch gemodificeerde LL leidt tot antigen specifieke tolerantie. Deze aanpak zou zeer goed gebruikt kunnen worden in de ontwikkeling van effectieve therapieën voor verscheidene auto-immuun, inflammatoire en allergische aandoeningen.

In hoofdstuk 4 draait het om de blokkade van een zeer belangrijk regulatoir cytokine, TGF-β middels een TGF-β 1 en 2 inhiberende peptide (p17). We laten zien dat dit specifieke peptide in staat is om in vitro en in vivo regulatoire T-cell activiteit te verlagen. Zo is p17 in staat om in ons model waarbij de genetisch gemodificeerde LL-OVA in OVA-geimmunizeerde DO11.10 muizen orale tolerantie induceert, dit op te heffen. Deze bevindingen benadrukken de belangrijke rol van TGF-β in ons model zowel als dat de TGF-β inhiberende peptide een waardevol middel zou kunnen zijn om vaccinatie werkzaamheid te vergroten als om tolerantie te verbreken tegen pathogenen of tumor antigenen.

Vervolgens hebben wij in hoofdstuk 5 een andere genetisch gemodificeerde bacterie gemaakt, die in staat is om HLA-DQ8 immuno-dominante epitopen uit te scheiden. Dit is een bestanddeel van gluten waarvan we weten dat coeliakie patiënten er erg gevoelig voor zijn. Deze bacteriën zijn specifiek gemaakt om tolerantie te induceren bij glutenovergevoeligheid, oftewel coeliakie. Om de effectiviteit van de door ons gegenereerde bacteriën te testen hebben we gebruik gemaakt van de HLA-DQ8 transgene NOD muizen, deze muizen hebben een humaan DQ8 MHCIi transgen, hetgeen ze extra gevoelig maakt voor de HLA-DQ8 epitopen in combinatie met een grotere predispositie voor auto-immuniteit in verband met hun NOD achtergrond. Hier zien we ook dat de orale administratie van de genetisch gemodificeerde LL-eDQ8d een lokale en systemische immuun suppressie geeft waarbij er een significante inductie is van Foxp3+ T cellen die waarschijnlijk door een gecombineerd IL-10/ TGF-β mechanisme werken. Deze resultaten ondersteunen de eerdere resultaten en daarmee de ontwikkeling van mucosaal geïnduceerde antigen-specifieke tolerantie voor de behandeling van zowel mucosale als systemische autoimmuun, inflammatoire of allergische ziektes, zo ook voor de zeer frequent voorkomend aandoening coeliakie.

In het volgende hoofdstuk, Hoofdstuk 6 demonstreren we de verschillen in immunogeniciteit tussen Balb/c en BL/6 muizen (Th1 versus tolerogeen). Hieruit blijkt dat in verband met het initiële Th1 adjuvant effect van LL in Balb/c muizen, deze muizen ongeschikt zijn voor de evaluatie
van systemische tolerantie inductie in profylactische settings van Th1 dominante ziektes. Verder zien we dat in langere voedingsregimes het Th1 adjuvant effect bij Balb/c muizen afneemt. Aan de andere kant laten onze data verkregen in therapeutisch settings, gebruik makend van een Th1 gedreven OVA inflammatie model zien dat het Th1 adjuvant effect de inductie van regulatoire T-cel respons in antigen-gesensitizeerde muizen niet in de weg staat. In deze studie laten we het belang zien van ‘herkenning’ van het antigen (de muis heeft de LL nog nooit gezien en de mens wel) in combinatie met specifieke immunogeniciteit van verschillende muismodellen en benadrukken we de voorzichtigheid die geboden moet worden met de extrapolatie naar de mens.

Hoofdstuk 7 is een review over regulatoire T-cellen in IBD. Hier omschrijven we dat bij IBD geactiveerd mucosale T cellen geremd kunnen worden door verschillende regulatoire T-cel subsets, welke steeds beter functioneel en phenotypisch gekarakteriseerd kunnen worden. Ze kunnen gegenereerd worden in de thymus en in perifere weefsels, daarbij resulteert, in muizen, een verlies van regulatoire T-cel activiteit in IBD. Helaas is tot op heden nog weinig bekend over de exacte mechanisme en functie van humane regulatoire T cellen in IBD. Tevens is er nog te weinig bewijs voor een vermindere regulatoire T cel functie in IBD. Hooggevend is dat regulatoire T-cellen gegenereerd of geactiveerd kunnen worden in vitro en in vivo gebruik makend van verschillende aanpakken, zoals specifieke ‘small molecules’, cytokine-gemedieerde activatie, danwel gen therapie. In preklinische setting hebben regulatoire T cellen therapeutische activiteiten, de volgende stap is om dit om te zetten in effectieve humane therapieën.

Ons specifieke onderzoek bestaande uit de inductie van antigeen-specifieke orale tolerantie door de genetisch gemodificeerde LL heeft veel publiciteit gehad. In hoofdstuk 8 vindt U de editorial in Gastroenterology en een paar hoogtepunten uit de pers.

In conclusie verschaft dit proefschrift, hoofdstuk 9, een degelijke basis en belangrijke data dat LL gebruikt kan worden voor het efficiënt afgeven van antigenen voor de mucosale inductie van antigeen-specifieke perifere tolerantie. Deze manier van administratie induceert efficiëntere immuunresponsen dan gezien wordt met gepurificeerd antigen en omzeilt de problematiek omtrent grootschalig eiwit purificatie systemen. Adequate biologische veiligheids strategien en efficiënte antigen afgifte maken LL een zeer attractieve kandidaat voor de inductie van specifieke orale tolerantie tegen bekende antigenen. Daarbij geeft deze aanpak hoop
voor nieuwe therapeutische interventies voor de behandeling van zowel mucosale als systemische autoimmuun, inflammatoire en/of allergische ziektes door specifieke antigen-secreterende *Lactococcus lactis*.
Curriculum vitae
Curriculum vitae

Inge Louise Huibregtse was born in Amsterdam, the Netherlands September 13th 1976. She obtained her Degree of Medical Doctor at the University of Amsterdam in 2003. During her medical training she followed several education programs in Italy and a 6-month voluntary training in basic research in the laboratory of Pediatric Gastroenterology and Nutrition at the New England Medical Center in Boston, USA under the supervision of Prof. Dr. R. J. Grand. She was involved in the research program on the molecular regulation of the human lactase-phlorizin hydrolase promoter. At present she is preparing a thesis on the induction of oral tolerance as a therapy for intestinal inflammatory diseases under guidance of Prof. Dr. S. J. H. van Deventer at the research laboratory of the department of Gastroenterology of the Academic Medical Center (AMC), Amsterdam. She studied two periods abroad: a 4-month period at the Flemish Institute for Biotechnology, Ghent, Belgium for the construction of genetically modified Lactococcus lactis and a 3 month period at the Mayo Clinic, Rochester, Minnesota, USA for testing genetically modified L. lactis in a genotypic Celiac Disease mouse model. Currently she is performing her clinical training in Gastroenterology in the Hospital, OLVG, Amsterdam and the Academic Medical Center, Amsterdam, the Netherlands.
Dankwoord
Dankwoord

Het onderzoek dat geleid heeft tot dit proefschrift is uitgevoerd op verschillende afdelingen en laboratoria:

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Het Vlaamsch Instituut voor Biotechnologie (VIB) in Gent, de afdeling Immunologie van de Mayo Clinic in Rochester, MN, U.S.A. en het Center for Applied Medical Research (CIMA) in Pamplona, Spanje.

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Sinds ik op het AMC meeloop, waak jij over me. Op cruciale momenten was jij er altijd om me te helpen. Ik ben heel erg blij dat jij mijn promotor wilt zijn en verheug me om op het feest de dans met jou te openen.

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Prof. Dr. S.J.H. van Deventer, beste Sander
In 2003 vertelde je iedereen: “Inge en ik gaan coeliakie oplossen.” Met dat vertrouwen in mij en met jouw briljante ideeën, waarvoor ik aanvankelijk op m’n tenen moest lopen om ze te begrijpen, is dit proefschrift begonnen.
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Prof. Dr. M.L.Kapsenberg, beste Martien
Zo’n goede wetenschapper en zo bescheiden, wat had ik zonder jullie gemoeten?

De leden van de beoordelingscommissie:
Prof. Dr. H.S.A. Heijmans, beste Hugo
Mijn grootmoeder was kinderarts en met haar als groot voorbeeld meldde ik me nog in mijn studententijd bij jou. Op mijn vraag wat ik zou moeten doen voor een opleidingsplaats Kindergeneeskunde, antwoordde jij: Laten zien hoe goed je bent!
Jij regelde voor mij een stage op het laboratorium van Prof. Richard Grand in Boston. Een betere plaats om kennis te maken met laboratorium onderzoek is er niet. Mijn grote plezier in onderzoek dateert uit die tijd. Nu, jaren later ben ik weliswaar in opleiding tot maag-, darm- en leverarts, maar het laboratoriumwerk houden we erin. Dat jij deel uitmaakt van mijn promotiecommissie, is voor mij heel bijzonder!

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Wat een eer dat jij me geholpen hebt bij mijn onderzoek. Ik blijf graag op de hoogte van jouw “koelbakje” en kijk uit naar het moment dat jij degene bent die coeliakie werkelijk gaat oplossen!

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Jij was natuurlijk de meest kleurrijke noot tijdens mijn promotie onderzoek. Uitstapjes naar Gent, naar Londen, de ruimte in, Biologie Beter, Groningen en niet te vergeten de werkbesprekingen in de Basiliek. Met mijn bewondering voor je scherpe intellect en grote kennis, dank ik je voor je onvoorwaardelijke hulp.

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Dr. J.N.Samson, beste Janneke
Vanaf het begin heb je me enorm geholpen met de muizen, met het DO11.10 muismodel, het testen van mijn LL-OVA sup en het uitwisselen
van protocollen. Ik ben dan ook erg blij dat je nu in mijn promotie commissie zit.

**Prof. Dr. R.P.J. Oude Elferink** wil ik ook heel graag danken als lid van mijn promotiecommissie.

Net zoals het leven, begon mijn promotieonderzoek in de baarmoeder. Tijdens mijn onderzoek in Gent moest ik er op een steenkoude winterdag op uit om aan een levend poelje (kip) te komen. Na een lange, moedeloze zoektocht over het uitgestorven Vlaamse platteland, slaagde ik er tenslotte in om in Kruishoutem voor 10 euro een kip te kopen. In een kartonnen doos nam ik de kip mee naar het “labo”, waarna ik een conventionele uterusextirpatie (baarmoederverwijdering) uitvoerde. Dit was de enige manier om het naar later bleek zeer waardevolle OVA RNA te kunnen isoleren. Hiermee was de kop er letterlijk en figuurlijk af en was binnen korte tijd mijn eerste genetisch gemodificeerde bacterie, de *Lactococcus lactis* OVA een feit.

Dit alles gebeurde in het **“labo” van het Vlaams Instituut voor Biotechnologie (VIB) in Gent**, en graag wil ik hierbij de medewerkers bedanken, met name **Koen van Laer** voor zijn hulp bij de ontwikkeling van mijn bacteriën en **Veerle Snoeck** voor het onderzoek dat we samen deden en voor de artikelen die daaruit voort kwamen.

Voor het testen van mijn bacteriën in verschillende muismodellen ging ik weer terug naar het **Centrum voor Experimentele en Moleculaire Geneeskunde (CEMM) in het AMC**. Proef na proef werd ingezet, hetgeen allemaal niet mogelijk was geweest zonder de nooit aflopende hulp van **Marieke ten Brink** en **Joost Daalhuizen**. **Henri Braat** introduceerde mij op L3, werkte mij in, begeleidde me en samen schreven we artikelen.

Verder wil ik iedereen van het **IBD lab** onder leiding van **Daan Hommes** en **Pieter Stokkers** bedanken voor hun hulp tijdens mijn onderzoek, evenals de andere CEMM labs, **alle CEMM analisten, biotechnici, het secretariaat** met in het bijzonder **Heleen**, als ook het **ICT personeel**.

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Het bijzondere muismodel, dat ik nodig had om mijn bacteriën te testen, bleek aanwezig in de Mayo Clinic in Rochester, Minnesota, U.S.A. Onder leiding van Dr.J.A.Murray kreeg mijn onderzoek daar een boeiend vervolg.

Dear Dr. Murray,
At the completion of my thesis I would like to thank you again for the highly instructive time I spent at your department. Besides your excellent supervision, your enthusiasm and encouragements, I will always remember the most enjoyable Thanksgiving Dinner with your family, which, from the very start made me feel welcome and at home in Rochester. Furthermore I would like to thank Eric and Shadi for the superb co-operation and everybody of Dr. Robin Patel’s lab, the Microbiology Research Facility of the Mayo Clinic for all their expertise, their support and for the great time we had together.

My scientific journey continued and I was introduced to Dr. J.J.Lasarte of the University of Navarra, Pamplona, Spain. Dear Juanjo, Although we never met, I want you to know how much I enjoyed our co-operation by email, resulting in the beautiful Journal of Immunology paper.

My Gastroenterology paper was accepted and as icing on the cake Dr. M.H.Maillard and Dr. S.B.Snapper from Harvard Medical School, Boston, U.S.A. wrote an editorial about my research. Thank you very much for the approval to use it as a chapter in my thesis.

Het promotie onderzoek bracht ook het bezoeken van congressen met zich mee, het presenteren van posters en het houden van praatjes. Met een groepje geweldige onderzoekers van het AMC reisden we regelmatig de wereld over en zagen New Orleans, Los Angeles, Washington, San Diego, Keystone tot Kiruna (Lapland), Kopenhagen, Capri, Genève, Parijs en Praag. Maar ook in het AMC waren er alle mogelijkheden om ook met onderzoekers van andere afdelingen van gedachten te wisselen, dan troffen we elkaar in het Oude Gasthuys, in de Epstein bar en bij de Albert Heijn-To-Go.

De afdeling Maag-, Darm- en Leverziekten op het AMC met mijn vader en Joep Bartelsman is altijd een soort thuisbasis voor me geweest. Mijn hele studietijd kon ik er terecht voor koffie, een praatje en een sigaret en voor mijn wasgoed.
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Met Floor erbij zijn we nu nog completer, onze bewonderenswaardige Juf Floor, onze parel op de koude bank, ik ben trots dat jij mijn paranimf bent.

Het thuisfront:
Er is geen dag van het hele traject voorbijgegaan, dat ik mijn ouders niet belde om verslag uit te brengen. Niemand heeft zo intens meegeleefd met mijn pieken en dalen als zij, mijn pad geëffend door geruisloos al mijn praktische problemen op te lossen en wensen te verwezenlijken. Of het nou ging om het opwachten van mijn loodgieter, het invullen van mijn belastingpapieren of een verblijf in Boston, een auto voor Gent, een strakke outfit voor de plenaire sessie in Kopenhagen, of het spetterende omslag van dit boekje (waarvoor ook veel dank aan Ad Petersen), Lieve P & M, hoe kan ik jullie ooit genoeg bedanken? Natuurlijk wil ik dit boekje ook aan jullie opdragen.

“STROMENDE BERGBEKEN, GEVULDE KOEKEN EN BLAUWE ZWAAILICHTEN” is de titel van mijn nieuwe boek, waarin ik met de liefste van de hele wereld in een droomscenario terecht ben gekomen en m’n geluk niet opkan.

Mijn lieve Flop, wat gaan we samen een heerlijke tijd tegemoet!
De roman speelt zich af in de Pijp en gaat over een ijzersterk team, een dokter en een kok, waarin daarnaast een grote rol is weggelegd voor alle vrienden die ik in de afgelopen tijd verwaarloosd heb. Dank jullie allemaal wel!


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List of publications
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