Interleukin 10 gene therapy for Crohn's disease
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Chapter I

Introduction

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Chapter I

Introduction

Crohn’s disease

Crohn’s disease is an inflammatory chronic disorder of the digestive tract that is usually localized in the terminal ileum and the colon. Clinical features consist of abdominal pain, diarrhea and weight loss, and the disease typically starts in early adult life. The incidence of Crohn’s disease has increased in the past decades, and the current prevalence in the northern part of the world is 0.5-0.8%. Crohn’s disease and ulcerative colitis are considered as the inflammatory bowel diseases (IBD). Ulcerative colitis differs from Crohn’s disease with regard to genetic background, immunological and clinical features and this condition is not further addressed in this thesis. In this introduction we will discuss briefly the present views on etiology and treatment of Crohn’s disease. The current notions on the most important cellular and molecular interactions that are involved in physiological immune responses will be considered in more detail, followed by the role of the immuno-regulatory cytokines, interleukin (IL)-10 and transforming growth factor β (TGF-β), and regulatory T cells in the intestine. Such knowledge is indispensable to understand the rationale for IL-10 based gene therapy in Crohn’s disease, which is the subject of this thesis.

Etiology of Crohn’s disease

Accumulating evidence suggests that Crohn’s disease represents the outcome of three interactive factors: host susceptibility, enteric microflora and the mucosal immune system. Host susceptibility is determined by environmental and genetic factors. Potentially relevant environmental factors include smoking and diet. Genetic factors associated with IBD have been extensively studied and the first susceptibility gene for Crohn’s disease has recently been identified. Mutations in the NOD2 gene, which encodes a protein with homology to plant disease resistance gene products, were found in approximately 40% of Crohn’s disease patients. Homozygosity for a functionally relevant mutation within this NOD2 gene confers an extremely high risk for the development of Crohn’s disease, and is considered to be the causative genetic defect in about 15% of the patients. It is tempting to speculate that NOD2 is involved in the
defense against certain luminal bacteria: it is expressed by antigen presenting cells such as monocytes and dendritic cells (Braat H, unpublished results) monocytes and, following stimulation by bacterial lipopolysaccharides (LPS), activates nuclear factor (NF-κB, a family of signal transduction proteins that regulates expression of many genes (i.e. TNF-α and IL-1β) involved in immune and inflammatory responses. It is possible that early recognition of (pathogenic) bacteria is defective in Crohn’s disease patients with mutant NOD2, leading to an abnormal T cell mediated response, tissue inflammation and aberrant cytokine production. However, the precise functional consequences of the NOD2 mutations that are involved in the pathogenesis of Crohn’s disease are not clear and need to be further characterized.

Many experimental and clinical studies have demonstrated that the enteric microflora is an essential cofactor in driving mucosal inflammation: germ-free mice do not develop experimental colitis, and diversion of the fecal stream following formation of a loop ileostomy has a beneficial effect in patients with Crohn’s disease. More direct evidence comes from studies by Duchmann et al, who showed that mucosal, but not peripheral blood, CD4+ T cells from Crohn’s disease patients proliferate when exposed to autologous intestinal bacteria.

The mucosal immune system is activated upon exposure to a pathogenic microorganism. Initially, innate immune cells, such as macrophages, dendritic cells and natural killer cells are recruited to eliminate pathogens by uptake and destruction or degradation. Macrophages and dendritic cells are activated and can present the products of pathogen degradation to antigen reactive T cells resulting in an adaptive immune response. T cells, in particular CD4+ T cells, play multiple roles as effector and regulatory cells in intestinal inflammation: a subset of naïve CD45RB+ CD4+ T cells has been shown to induce experimental colitis after transfer to severe combined immunodeficient (SCID) mice. In addition, the number of activated mucosal T cells secreting pro-inflammatory cytokines that mediate interactions between cells, such as interferon γ (IFN-γ), is increased in active Crohn’s disease. Finally, CD4+ cell depletion ameliorates inflammation in experimental colitis and in Crohn’s disease. By comparison, whereas CD8+ cells are important in first line defence in the epithelium, they do not appear to play a decisive pathologic role in intestinal inflammation.
Chapter I

Based on these observations, the current working model of the pathophysiology of Crohn's disease is that of an uncontrolled abnormal immune response of CD4+ cells to enteric bacteria in genetically susceptible individuals.

Therapy of Crohn's disease

Currently there is no curative treatment for patients with Crohn's disease. For several decades corticosteroids were the only potent anti-inflammatory agents available for treatment of active Crohn's disease. Corticosteroids act rapidly and have a wide range of anti-inflammatory activities, but do not alter the natural course of the disease; they do not consistently heal intestinal ulcers, and are associated with many side effects. The use of immunomodulatory therapy, as azathioprine and methotrexate has provided alternative therapeutic options. However, administration of these agents is limited by a relatively slow onset of action, inconstant efficacy, inadequate selectivity and substantial short and long-term toxicity. At present, more than 50% of patients will require surgical intervention at some point during the course of their disease to treat complications such as stricture, fistula, abscess or medically intractable disease.

New biological therapeutic strategies are based on the increased knowledge of the pathophysiology of Crohn's disease. Biological agents include recombinant cytokines, cytokine receptor antagonists, chimeric and humanized antibodies, antisense oligonucleotides and viral as well as non-viral vectors for gene transfer. Presently, of this growing range of biologic therapies, antagonism of TNF-α with the monoclonal TNF-α antibody infliximab is the only approach that has been approved for clinical use. Infliximab is effective and well-tolerated in patients with moderate to severe Crohn's disease that is resistant to conventional treatment and can maintain remission up to 44 weeks if administered by repeated intravenous infusions. The rapid healing of entero-cutaneous fistulas is a good example of the anti-inflammatory activity of infliximab in Crohn's disease. Traditional therapeutic agents have limited mucosal healing effects, and in the older studies endoscopic severity of ileocolonic Crohn's disease did not correlate well with therapeutic effects as measured by clinical parameters. However, the clinical improvement following infliximab therapy has been consistently associated with mucosal healing. The long-term efficacy and safety as well as the precise mode of
action of infliximab are currently under investigation. However, because of the immunogenicity of monoclonal antibodies and risk of side effects such as opportunistic infections and malignancy, infliximab may be best suited as a bridge to more long-term maintenance strategies. Recently, gene transfer has emerged as a new and promising method for maintenance of remissions in chronic inflammatory diseases, such as Crohn's disease, and we will here provide the rationale for the development of IL-10 based gene therapy.

**Physiological immune response to non-pathogenic antigens: peripheral tolerance**

Although the intestinal epithelium forms a physical barrier for pathogens, bacterial antigens are continuously sampled by gut mucosal dendritic cells, the major intestinal antigen presenting cells, and presented to T cells. The mucosal immune system needs to discriminate pathogens from dietary antigens and commensal bacteria in the gut lumen. Indeed, a large pool of immune cells resides within the mucosal epithelium and lamina propria. The continuous presence of potential pathogens in close proximity to immune cells must be tightly controlled to ensure that pathologic inflammation does not develop. Control is provided by mechanisms of tolerance. The concept of tolerance includes, by definition, any mechanism by which a potentially injurious immune response is prevented, suppressed, or shifted to a non-injurious class of immune response.

Central tolerance refers to thymic deletion of self-reactive cells, which is not complete and self-reactive cells escape to the periphery. Several mechanisms are involved in maintaining peripheral tolerance, i.e. anergy (functional unresponsiveness), apoptosis (programmed cell death) and the involvement of tolerogenic dendritic cells or regulatory T cells (figure 1). These mechanisms can interfere during all phases of the immune response and are probably all necessary to control 1) self-reactive cells, 2) cells reactive to non-pathogenic microorganisms and 3) cells that become activated following a normal immune response to a foreign antigen.
Chapter I

Figure 1  Mechanisms of peripheral tolerance
A normal T cell response is triggered by the recognition of antigen and second signals. Multiple mechanisms may function to inhibit the expansion or effector functions (or both) of T cells. As discussed in the text, these mechanisms appear to be most important for the maintenance of peripheral tolerance to "self" and "non-pathogenic, non-self" antigens. APC: antigen presenting cell; CTLA-4: cytotoxic T lymphocyte-associated antigen 4; PD-I: programmed cell death 1; PDL: PD-I ligand. FasL: Fas ligand; MHC: major histocompatibility complex; TCR: T cell receptor.

Physiological immune response to pathogens
The intestinal mucosa contains a large number of dendritic cells for uptake, processing and transport of antigens to the regional lymph nodes. Dendritic cells critically steer immune responses through interactions with T cells, requiring close proximity and crosstalk of these two cell types. The decision between immunity and tolerance is taken by dendritic cells: they induce the development of naïve T cells into effector or regulatory T cells, or can induce anergy/apoptosis of T cells.18-40

Dendritic cells express pattern recognition receptors (e.g. Toll like receptors)41 that recognize pathogen-associated molecules (e.g. LPS) from invading microbes. Such activation of pattern recognition receptors generally increases the immune-stimulatory capacities of dendritic cells. After antigen uptake, activated and maturing dendritic cells
Introduction

start to express the chemokine receptor CCR7. Chemokines are a family of leukocyte chemoattractants that regulate both inflammatory cell recruitment and homeostatic trafficking of leukocytes. Dendritic cells migrate to the regional lymph nodes, where specialized high endothelial venules (HEVs) express the CCR7 ligand CCL2.\textsuperscript{42} CCR7 is also present on naïve T cells\textsuperscript{43, 44} and directs, together with the lymph node homing receptor L-selectin (CD62L),\textsuperscript{45} the migration of naïve T cells to the regional lymph nodes. The passage across the HEVs is a multistep process that involves selectin-supported rolling, followed by a triggering event, and firm integrin-mediated adhesion.\textsuperscript{46, 47}

When naïve T cells encounter their cognate antigen presented by a dendritic cell in a lymph node, they differentiate into effector/memory CD4\textsuperscript{+} or cytotoxic CD8\textsuperscript{+} T cells. Based on their cytokine secretion profile, CD4\textsuperscript{+} T cells differentiate into at least 2 subsets of helper cells, T helper 1 (Th1) and T helper 2 (Th2) cells (figure 2).\textsuperscript{48} Th1 cells produce the pro-inflammatory cytokines IL-2 and IFN-γ and protect against intracellular pathogens (cellular response), whereas Th2 cells selectively secrete IL-4, IL-5 and IL-13, and counter extracellular pathogens through production of antibody by B cells (humoral response). Both subsets of T helper cells can also produce the two pivotal immunoregulatory cytokines, IL-10 and TGF-β. However, regulatory T cells, which are discussed more fully below, are the main producers of these cytokines.

The cytokine microenvironment to which naïve T cells are exposed is the primary determining factor for the differentiation towards a Th1 or Th2 cell type:\textsuperscript{49} IL-12 with support of IL-18, both produced by antigen presenting cells, induce Th1 differentiation\textsuperscript{50, 51} and IL-4, produced by natural killer cells, mast cells, basophils and mature CD4\textsuperscript{+} cells,\textsuperscript{52} drives Th2 differentiation.\textsuperscript{53} Others factors such as antigen density,\textsuperscript{54} major histocompatibility complex (MHC) haplotypes,\textsuperscript{55} type of antigen presenting cell,\textsuperscript{56} and costimulatory factors\textsuperscript{57} also influence the selective development of Th1/Th2 cells.
Chapter 1

![Diagram of T helper cells and their functions](image)

**Figure 2 Induction and regulation of T helper 1 and T helper 2 cells**

In the standard model, T helper (Th) cells can differentiate into Th1 or Th2 depending on the cytokine micro-environment. Interleukin (IL)-12 drives Th1 cells, whereas IL-4 promotes Th2 cells. Interferon γ (IFN-γ) and IL-4, produced by Th1 and Th2 cells respectively, can also act as autocrine growth factors as well as inhibitory factors for the opposite subset. Th1 cells enhance IgG2a synthesis by B cells through IFN-γ, whereas Th2 cells induce B cell IgE and IgG1 production through IL-4. Functionally, Th1 cells promote cell-mediated immunity and host-defense against intracellular pathogens, but also contribute to the pathogenesis of diseases such as Crohn's disease, rheumatoid arthritis and psoriasis. Th2 cells attenuate cell-mediated immunity but promote anti-helminth responses, and increase allergic reactions through IL-4, IL-5 and IgE.

R: receptor; TCR: T cell receptor, NK: natural killer cell.
Introduction

After local priming of T cells in the presence of high levels of IL-12 and IL-18, the differentiated effector Th1 cells and memory T cells recirculate preferentially to the intestinal mucosa. The integrin α4β7, expressed on these T cells, is the principal gut homing receptor and functions at several steps in the adhesion cascade by interacting with the mucosal addressin MAdCAM-1 present on endothelial cells. In addition, the chemokine TECK and its receptor CCR9 are implicated in the migration of α4β7 memory T cells to the small intestine. Once arrived in the intestine the Th1 effector cells mount an inflammatory response on encounter of the antigen, and this response can be amplified by freshly recruited immature dendritic cells (figure 3).

After elimination of the pathogen, the bulk of the effector T cells become redundant and most of them disappear, in large part through apoptosis. However, a small proportion survives to become long-lived memory cells. Thus, whereas induction of anergy and apoptosis serve to reduce the number of T cells that can respond to mucosal antigens, this mechanism is not sufficient for elimination of all potentially reactive T cells. Cells that escape these mechanisms of peripheral tolerance need to be controlled by tolerogenic dendritic cells and/or regulatory T cells, which are discussed below.

IL-10 and TGF-β

IL-10 and TGF-β are now well recognized as the two main immuno-regulatory cytokines. Human IL-10 is an 18 kDa polypeptide, that shares significant sequence homology to mouse IL-10 (73% amino acid homology). Mouse IL-10 is specific for mouse cells, but human IL-10 can activate both human and mouse cells. IL-10 is produced by T cells, monocytes/macrophages, dendritic cell, keratinocytes and B cells, usually in response to an activation stimulus. The IL-10 receptor (R) is composed of at least two subunits that are members of the interferon receptor family: IL-10R1 (expressed on most hemopoietic cells), and the coreceptor IL-10R2 (constitutively expressed on most cells). IL-10 is a multifunctional cytokine with effects on most hemopoietic cells (functions are summarized in table 1). IL-10 inhibits a broad array of immune parameters, including activation and effector function of T cells, monocytes and macrophages, limiting and ultimately terminating inflammatory responses.
### Chapter 1

#### Table 1 | Biological functions of interleukin 10

<table>
<thead>
<tr>
<th>Celltype</th>
<th>↓ Inhibition</th>
<th>↑ Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monocytes/</td>
<td>Production of pro-inflammatory cytokines and chemokines</td>
<td>Expression of IL-1 receptor antagonist, IL-1RI and IL-1RII, soluble p55 p75 TNFR, LPS receptor (CD14), Fcγ receptors</td>
</tr>
<tr>
<td>Macrophages</td>
<td>Expression of chemokine receptors and Toll like receptor 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antigen presentation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prostaglandin E2, superoxide anion and nitric oxide production</td>
<td></td>
</tr>
<tr>
<td>Dendritic cells</td>
<td></td>
<td>Apoptosis</td>
</tr>
<tr>
<td></td>
<td>IL-12</td>
<td>Decoy receptors for inflammatory chemokines in presence of pro-inflammatory signals and IL-10</td>
</tr>
<tr>
<td></td>
<td>antigen presentation</td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>Via APC: pro-inflammatory cytokine production and proliferation</td>
<td>Recruitment, cytotoxic activity and proliferation of CD8⁺ cells</td>
</tr>
<tr>
<td></td>
<td>Direct: expression of IL-2, TNF-α, IL-5 by CD4⁺ cells</td>
<td>Direct: IFN-γ production by CD8⁺ cells</td>
</tr>
<tr>
<td>NK cells</td>
<td>via APC: IFN-γ production</td>
<td>Anergy of T cells</td>
</tr>
<tr>
<td>B cells</td>
<td>MHC class II</td>
<td>Expression of TGF-βRII on activated T cells</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Production of pro-inflammatory cytokines and chemokines</td>
<td>Survival, proliferation, antibody secretion (IgA, IgG)</td>
</tr>
</tbody>
</table>

Viral IL-10, a product encoded by Epstein-Barr virus, is highly homologous to both human (84% amino acid homology) and mouse IL-10 and they share many biological properties. However, in contrast to human and mouse IL-10, viral IL-10 enhances MHC class II expression on B cells, does not effectively costimulate thymocyte or mast cell proliferation and has a reduced ability to inhibit IL-2 production by activated CD4⁺ cells in vitro.

TGF-β is a 25 kDa protein that is produced by all leukocyte lineages, including lymphocytes, macrophages and dendritic cells. In mammals, TGF-β exists in three isoforms, TGF-β1, TGF-β2, and TGF-β3 that have a high degree of amino acid homology. One or more of the TGF-β receptors or ligands are expressed in practically
every tissue of the body. The three isoforms have similar effects *in vitro*, but differential expression during embryogenesis is reflective of the distinct functions. TGF-β is capable of regulating growth, proliferation and differentiation, extracellular matrix formation and function of immune and non-immune cells. TGF-β modulates the expression of adhesion molecules, provides a chemotactic gradient for leukocytes and other cells participating in an inflammatory response, and inhibits these cells following activation.

**Role of IL-10 and TGF-β* in vivo**

The potent immuno-regulatory properties of TGF-β are illustrated in TGF-β1 mice that develop a severe, multifocal inflammation shortly after birth (lethality 50% in TGF-β1 mice and 100% in TGF-β2 and TGF-β3 mice). On the other hand, overexpression of TGF-β can also be lethal or depending on the promoter induce tissue specific pathology that may include fibrosis. Thus, tight regulation of TGF-β appears absolutely critical for survival.

In contrast with the broad anti-inflammatory activities of TGF-β, the regulatory activities of IL-10 seems to be of particular importance in the intestine. IL-10 and II.-10 receptor mice develop a chronic enterocolitis resulting in growth retardation and anaemia. The development and severity of the colitis are a consequence of an inappropriate immune response to intestinal antigens. It is uncertain whether the difference between TGF-β and IL-10 represents a true compartmentalisation of IL-10 function or whether TGF-β compensates for IL-10 in IL-10 mice in all organs except the intestine. Evidence that TGF-β functions via IL-10 in the intestine is provided by Kitani et al, who showed that plasmids encoding TGF-β inhibited a Th1-mediated experimental colitis and that this was at least in part due to IL-10 induction and subsequent suppression of II.-12.

Studies in IL-10 transgenic mice, in which IL-10 cDNA was regulated by different promoters, indicated that IL-10 can mediate immunosuppressive or immunostimulatory activities *in vivo* depending on the type of immune response and on the type of cell expressing II.-10. On the other hand, TGF-β has toxic effects at therapeutic doses: systemic administration of high doses of recombinant (r)TGF-β1 to rats and rabbits produced a spectrum of lesions in multiple tissues. Moreover, therapeutic use is
Chapter I

hampered by the extremely short half-life of rTGF-β (< 11 min). By contrast, systemic administration of rIL-10 to mice is safe and has therapeutic efficacy in different models of experimental colitis. In the only clinical trial to examine the effect of the systemic administration of a rTGF-β2 in patients with multiple sclerosis, significant but reversible declines in renal function were observed, probably due to vasoconstriction and fibrosis of the renal vasculature. Thus, in experimental as well as clinical studies, systemic use of rTGF-β is limited by its adverse effects.

Another cytokine considered to have immunoregulatory functions is IL-4, which promotes development of Th2 cells and inhibits development Th1 cells. The role of IL-4 in the intestinal inflammation is less clear than the role of IL-10: IL-4 mice show no signs of colitis. In one study, administration of an adenoviral vector containing the IL-4 gene to rats with TNBS colitis reduced inflammation, but rIL-4 treatment did not suppress or even exacerbated the development of colitis in the CD45RB<hi>CD4</hi> transfer model. At present, IL-4 is clinically used for its antitumor activity.

These data indicate that both IL-10 and TGF-β, but not IL-4, play an important role in mucosal homeostasis in the intestine. However, in view of the numerous actions of TGF-β, the ubiquitous expression of TGF-β and its receptors, and the adverse effects of systemic rTGF-β, and the relative specific regulatory function of IL-10 within the mucosal immune system, the latter cytokine seems a more attractive candidate for therapeutic application in intestinal inflammation.

Types of regulatory T cells

Several studies in models of experimental colitis indicate that regulatory CD4<hi>T</hi> cells play a key role in intestinal peripheral tolerance. Besides regulatory CD4<hi>T</hi> cells, other regulatory cells including CD8<hi>T</hi> and CD4<hi>CD8</hi> T cells have been described, but their role in the intestine is less clear and they will not be discussed here. Regulatory T cells have been described as precommitted precursor cells, produced in the thymus, but others believe that they are differentiated in the periphery following encounter of tolerogenic stimuli. These are two non-mutually exclusive hypotheses and a third unified hypothesis is that they belong to the same subset of cells in different stages of
Introduction

their differentiation. Regulatory CD4<sup>+</sup> cells have been studied in various in vitro and in vivo model systems, and we will focus on data relevant for intestinal inflammation.

Regulatory CD4<sup>+</sup> cells that express the IL-2 receptor α chain (CD25), are derived from the thymus. They naturally occur both in mice<sup>1</sup> and in humans<sup>2</sup>. CD25<sup>+</sup> CD4<sup>+</sup> cells are hyporesponsive to stimulation, are capable of production of low levels of IL-10 and perhaps TGF-β (varies among reports), and they were shown to suppress the response of CD25<sup>−</sup> CD4<sup>+</sup> cells to stimulation in vitro. Murine CD25<sup>+</sup> CD45RB<sup>+</sup> CD4<sup>+</sup> cells were able to prevent experimental colitis induced in SCID mice by the CD45RB<sup>+</sup> CD4<sup>+</sup> cells.<sup>3</sup>

Three types of regulatory T cells are described that are believed to differentiate from naïve T cells in the periphery upon encounter of antigen. The first type was identified by Groux <i>et al.</i>, who used repeated antigenic stimulation of murine and human CD4<sup>+</sup> cells in the presence of IL-10 to generate so called Tr1 cells.<sup>4</sup> Tr1 cells have a low proliferative capacity, produce large quantities of IL-10, some TGF-β, low levels of IL-2 and no IL-4. These antigen-specific T cell clones suppressed the proliferation of CD4<sup>+</sup> cells in response to antigen in vitro<sup>5</sup> and prevented experimental colitis induced in SCID mice by transfer of CD45RB<sup>+</sup> CD4<sup>+</sup> cells.<sup>6</sup>

The second type, the so called Th3 cell, has been generated in vitro in the presence of TGF-β, IL-4, IL-10 and anti-IL-12 from naïve CD4<sup>+</sup> cells isolated from T cell receptor (TCR) transgenic mice.<sup>7</sup> These Th3 cells primarily secrete TGF-β, and have also been described to naturally occur in chronic human helminth infection.<sup>8</sup> T cells with a similar Th3 phenotype have been reported to arise in the intestinal mucosa of mice fed with the oral antigen trinitrophenol (TNP)-haptenated colonic protein (HCP).<sup>9,10</sup> The presence of Th3 cells prevented the development of trinitrobenzene sulfonic acid (TNBS) colitis, induced by intrarectal administration of the haptenating agent TNBS.<sup>11</sup>

The third type of regulatory T cell is induced by repetitive stimulation of human naïve cord blood-derived CD4<sup>+</sup> cells with immature dendritic cells.<sup>12</sup> Similar to thymus-derived regulatory T cells, these cells express CD25, and are hyporesponsive to stimulation. However, they exclusively produce high levels of IL-10 and have the functional capacity to inhibit proliferation of Th1 cells in vitro.<sup>13</sup>
Chapter 1

Hence, in recent years it has become clear that different types of regulatory T cells functionally inhibit activation and proliferation of potentially "pathogenic" effector T cells in vitro and in experimental colitis.

Mechanism of action of regulatory T cells

Both cell contact dependent-signals\(^1\) and secretion of immunoregulatory cytokines IL-10 and TGF-β\(^1\) are thought to be involved in the function of regulatory T cells. Cell contact-dependent suppression is mediated by the cytotoxic T cell-associated antigen-4 (CTLA-4). CTLA-4 is a cell surface molecule that binds to the costimulatory molecules CD80 and CD86 present on antigen presenting cells and downregulates T cell activation.\(^2\) Inhibition of the CTLA-4 pathway, by using neutralizing antibodies, reduced the suppressor function of CD25\(^+\)CD45RB\(^-\)CD4\(^+\) cells in experimental colitis in SCID mice.\(^3\) The essential role for IL-10 in the function of regulatory T cells was demonstrated by the observation that colitis in SCID mice induced by CD45RB\(^{bsh}\)CD4\(^+\) cells was not prevented by CD45RB\(^{bsh}\) cells isolated from IL-10\(^-/-\) mice.\(^4\) The importance of TGF-β was demonstrated in SCID mice, reconstituted with pathogenic CD45RB\(^{bsh}\)CD4\(^+\) cells and protective CD45RB\(^{bsh}\)CD4\(^+\) cells that developed colitis when treated with anti-TGF-β antibodies.\(^5, 6\)

Expression of CTLA-4 and secretion of IL-10 and TGF-β by regulatory T cells may be linked. One possible mechanism for the linkage was provided by Chen et al, who demonstrated that cross-linking of CTLA-4 in the presence of TCR-mediated signals induces TGF-β secretion.\(^7\) In addition, a recent study not only showed that CTLA-4 enhanced TGF-β expression, but also that TGF-β present on the cell surface of regulatory cells inhibited proliferation (via binding to the TGF-β receptor) of responder CD25 CD4\(^+\) cells.\(^8\)

Moreover, IL-10 and TGF-β are functionally linked, since the protective effect of Th3 cells secreting TGF-β in TNBS colitis was abolished by administration of IL-10 neutralizing antibodies.\(^9\) Different, but not mutually exclusive possibilities exist to explain the related function of IL-10 and TGF-β. A first possibility is that IL-10 is necessary for responsiveness of cells to the regulatory effects of TGF-β. A recent study supported this view by showing that activated T cells manifested reduced TGF-βR.
expression in vitro and this effect was reversed by IL-10, facilitating the inhibitory function of TGF-β. Another study showed that IFN-γ could induce intracellular production of SMAD-7 (inhibitor of TGF-β signalling) in fibroblasts and thus interfered with intracellular TGF-β signalling. Because IFN-γ is downregulated by IL-10, TGF-β signalling is better maintained in the presence of IL-10. Another possibility is that decrease of Th1 cytokine production by IL-10, serves to block the negative effects of this class of cytokines on the expansion of TGF-β cells. However, it is possible that high amounts of IL-10 secreted by regulatory T cells also inhibit Th1 mediated inflammation in the absence of TGF-β. Whether regulatory T cells primarily inhibit activation, recruitment or effector function of their target cells still has to be elucidated.

In summary, the different types of regulatory T cells that are relevant for the work in this thesis seem to depend on cell-cell contact via CTLA-4-CD80/86 costimulatory molecules interaction and on the immuno-regulatory cytokines (membrane bound or secreted) IL-10 and/or TGF-β for their suppressive function in vivo.

The role of antigen in activation of regulatory T cells

In Crohn's disease as well as in experimental models of intestinal inflammation the identity of the inciting antigens is undefined, and the resulting T cell activation is polyclonal. In addition, the antigen-specificity of the regulatory T cells in the intestine is unknown. In fact, exposure to bacterial-specificity seems to be dispensable for the effector function of regulatory T cells both in vitro and in vivo, as CD45RB+CD4+ cells isolated from germ-free mice were able to inhibit colitis. It is also conceivable that the presence of the normal intestinal flora provides inflammatory signals, which drive the expansion of regulatory T cells. Moreover, it is possible that regulatory T cells react to heat shock proteins that are ubiquitously exposed at sites of inflammation, and limit inflammatory responses in general. Alternatively, regulatory T cells may be activated by an irrelevant antigen (for example ovalbumin in experimental models) and inhibit the function of T cells by a phenomenon termed 'antigen driven bystander suppression'.

The therapeutic potential of antigen-specific regulatory T cells has been shown in many animal models, including graft versus host disease, diabetes, autoimmune encephalomyelitis, autoimmune thyroiditis, and immediate hypersensitivity/allergy.
Chapter I

However, antigen-nonspecific regulatory T cells have not been previously used in chronic inflammatory conditions.

**What is wrong with the mucosal immune system in Crohn’s disease?**

Clinical and experimental studies have provided evidence for several defects of the mucosal immune response in Crohn’s disease. Based on the predominance of IL-2 and IFN-γ secreting mucosal T cells and the specific expression of the Th1 driving cytokines IL-12 and IL-18 in the intestinal mucosa, Crohn’s disease is considered to be a prototype Th1 disease.\(^\text{18, 121, 123}\) Apparently, mechanisms of peripheral tolerance are not effective in downregulating this Th1 cell mediated inflammation, and two studies have reported that mucosal T cells in patients with Crohn’s disease are resistant to apoptosis.\(^\text{124, 125}\) Our laboratory has recently demonstrated that the number of dendritic cells producing IL-12 and IL-18 in the intestinal mucosa is increased in patients with Crohn’s disease, indicating the enhanced activation of the immune system in the intestine (te Velde A, unpublished results).

In the intestinal mucosa of healthy individuals, CD4+ cells have been shown to contribute to downregulation of effector T cells, via production of IL-10 and TGF-β.\(^\text{126}\) Conflicting reports exists concerning suppressor/regulatory cell activity by lamina propria cells\(^\text{127, 128}\) and peripheral blood mononuclear cells\(^\text{129, 130}\) in Crohn’s disease patients. However, Crohn’s disease is characterized by a relatively reduced production of bioactive IL-10 in the lamina propria, and the resulting IL-10 concentrations seem insufficient to downregulate pro-inflammatory cytokines.\(^\text{131, 132}\) A low ileal IL-10 concentration is associated with early endoscopic recurrence of disease after surgery in patients with Crohn’s disease.\(^\text{113}\) Our laboratory has recently found that mucosal memory (CD45RB\(^{+}\)a CD45RO\(^-\)) CD4+ cells from patients with Crohn’s disease are less abundant compared with controls and produce less IL-10.\(^\text{134}\) Conversely, increased TGF-β1 expression was found in affected mucosa of patients with active Crohn’s disease,\(^\text{145, 150}\) and it was shown that mucosal T cells from IBD patients were insensitive to the
Figure 3 Immune response in Crohn's disease and therapeutic targets for biological agents

Under inflammatory conditions, migrating dendritic cells transport antigens to regional lymph nodes. Presentation of antigens to naïve T cells in the presence of IL-12 results in the generation of T helper 1 (Th1) effector cells that migrate into the intestine to mediate an inflammatory response. Targets for biological agents may be divided according to the different phases of an immune response: 1) antigen and antigen presentation, 2) activation of effector T cells, 3) cytokine-mediated response amplification, 4) adhesion and recruitment, 5) repair and restitution.

regulatory effects of TGF-β because they overexpress SMAD7, an inhibitor of TGF-β signalling. These data suggest that in Crohn’s disease several defects in the mechanisms of peripheral tolerance coexist, including resistance to T cell apoptosis and disrupted regulatory T cell function, that lead to a final common pathway of Th1 cell mediated intestinal inflammation.
Chapter 1

Recombinant IL-10 for Crohn's disease

Based on successful experimental findings showing the central role of IL-10 in maintaining mucosal homeostasis, the clinical benefit of rIL-10 administration was studied in patients with Crohn's disease. Four controlled randomized trials have been conducted to assess the efficacy of rIL-10 in patients with Crohn's disease. A one-week daily intravenous infusion of 0.5-25 μg/kg rIL-10 in patients with steroid-refractory active Crohn's disease (46 patients) was safe and well-tolerated. Although the study was not designed to assess efficacy, 50% of the rIL-10 treated patients versus 23% of the placebo patients had a complete clinical remission during the 3-week follow-up period.

Two subsequent trials investigated the safety and efficacy of subcutaneous administration of rIL-10 in mild to moderate and chronic active Crohn's disease patients (95 and 329 patients, respectively). In both studies systemic treatment for 28 consecutive days with either placebo or one of four doses of rIL-10 (1, 4 or 5, 8 or 10, and 20 μg/kg) had modest beneficial clinical effects. Data at 20-week follow-up suggested that the beneficial effects may be sustained beyond the period of active treatment. In patients responding to rIL-10, a decrease in activation of NF-κB (p65) was observed in ileal biopsy specimens. This effect could explain, at least in part, the ability of IL-10 to suppress the synthesis of pro-inflammatory cytokines, such as IL-1 and TNF-α in the intestinal mucosa. Remarkably, the beneficial effect in both studies of low-dose rIL-10 was lost at higher doses, resulting in a “bell-shaped” dose response curve, which was reminiscent of findings in experimental colitis and rheumatoid arthritis. The adverse effects of high-dose rIL-10 included mild flu-like symptoms (headache, fever, back pain and dizziness). A possible explanation for this phenomenon was the finding that in Crohn's disease patients as well as in healthy volunteers, high dose rIL-10 (e.g. 20 μg/kg) stimulates IFN-γ production resulting in immunostimulatory effects.

The fourth study was performed to evaluate safety, tolerance and prevention of endoscopic recurrence by rIL-10 treatment in patients operated on for Crohn's disease (65 patients). Compared with placebo, rIL-10 (4 or 8 μg/kg) was safe and well-tolerated during the twice-weekly subcutaneous administration for 12 weeks and during the 4-week follow-up. Although the power of the study was insufficient to evaluate...
Introduction

efficacy, no clear evidence of prevention of endoscopic recurrence of Crohn’s disease was observed.

The short serum half-life of rIL-10 (1.5-3 hours) that necessitates frequent administration of relatively large doses to achieve a therapeutic concentration in the target organ is a limitation of systemic administration of rIL-10. Systemic rIL-10 treatment in Crohn’s disease may result in mucosal IL-10 concentrations that are insufficient to downregulate inflammation and therefore lack efficacy. In addition, IL-10 administration may be only successful for preventing (and not treating) a disease as suggested by animal experiments.

Taken together, systemic rIL-10 treatment is safe and modestly effective in patients with Crohn’s disease. Systemic administration is not optimal, because of the side effects and rapid clearance that necessitates frequent administration. Either local administration or targeted gene delivery would offer the prospect of achieving higher local concentrations of the IL-10 gene for downregulation of the immune response in Crohn’s disease and preventing systemic undesirable effects.

Towards gene therapy for Crohn’s disease

Vectors expressing immunoregulatory genes may offer advantages in treatment of Crohn’s disease. Both viral and non-viral vectors have been applied to study gene delivery in cell cultures and in models of experimental colitis. A summary of these studies is listed in table 2. In addition, genetically modified bacteria can be used to locally deliver a therapeutic gene. Proof of principle of the latter idea was obtained in two models of experimental colitis, in which intragastric administration of a Lactococcus lactis secreting IL-10 prevented the development of colitis.

For IL-10 gene therapy, not only the choice of vector but also the choice of the target cell is important: IL-10 produced by an epithelial cell will have a different effect when compared with IL-10 produced by a T cell, a dendritic cell or a monocyte. The main features of gene therapy are reviewed in chapter 3 of this thesis. Suffice to mention here the many experimental studies in models of rheumatoid arthritis, multiple sclerosis, and diabetes that have provided a strong rationale for gene therapy of these immune-mediated diseases. The considerable experimental evidence for therapeutic
Chapter 1

Table 2 *In vitro* and *in vivo* gene therapy studies for intestinal inflammation

<table>
<thead>
<tr>
<th>Vector</th>
<th>Features</th>
<th>Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naked plasmid DNA/Liposome</td>
<td>➤ Potential delivery to wide range of cells</td>
<td>➤ Intranasally or i.m. TGF-β plasmid prevents exp. colitis(^\text{60,161})</td>
</tr>
<tr>
<td></td>
<td>➤ Transient expression</td>
<td>➤ Efficient infection of intestinal epithelial cells <em>in vitro</em>(^\text{162})</td>
</tr>
<tr>
<td></td>
<td>➤ Low efficiency of gene transfer</td>
<td>➤ IL-10 and IL-4 gene complexed to liposomes in Crohn’s disease(^\text{160})</td>
</tr>
<tr>
<td>Adenovirus (Adv)</td>
<td>➤ Infects wide range of cells</td>
<td>➤ Efficient infection of intestinal epithelial cells(^\text{163-165}) and dendritic cells (^\text{166}) <em>in vitro</em></td>
</tr>
<tr>
<td></td>
<td>➤ Targeting possible</td>
<td>➤ AdvIL-10 iv prevents exp. colitis(^\text{167,168,169})</td>
</tr>
<tr>
<td></td>
<td>➤ Transient gene expression</td>
<td>➤ Intrarectal AdvIL-18 antisense mRNA suppresses exp. colitis(^\text{170})</td>
</tr>
<tr>
<td></td>
<td>➤ High efficiency of gene transfer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➤ Host immune response</td>
<td></td>
</tr>
<tr>
<td>Adeno-associated virus (AAV)</td>
<td>➤ Infects wide range of cells and targeting possible</td>
<td>➤ Intragastric AAV expressing β-galactosidase prevents lactose intolerance in rats(^\text{171})</td>
</tr>
<tr>
<td></td>
<td>➤ Potential long-term expression</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➤ Low immunogenicity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➤ Small packaging capacity 4 kb</td>
<td></td>
</tr>
<tr>
<td></td>
<td>and difficult manufacturing</td>
<td></td>
</tr>
<tr>
<td>Retrovirus</td>
<td>➤ Infects only dividing cells</td>
<td>➤ Efficient infection of T cells(^\text{172}) and dendritic cells(^\text{173}) with a retroviral vector *in vitro*</td>
</tr>
<tr>
<td></td>
<td>➤ Potential long-term gene expression</td>
<td>➤ IL-10 transduced CD4(^\text{+}) cells prevent exp. colitis(^\text{174,175})</td>
</tr>
<tr>
<td></td>
<td>➤ High efficiency of gene transfer to dividing cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➤ No immunogenicity</td>
<td></td>
</tr>
<tr>
<td>Lentivirus</td>
<td>➤ Infects wide range of cells</td>
<td>➤ Efficient infection of T cells(^\text{176}) and intestinal epithelial cells(^\text{177}) with a lentiviral vector *in vitro*</td>
</tr>
<tr>
<td></td>
<td>➤ Potential long-term gene expression</td>
<td></td>
</tr>
<tr>
<td></td>
<td>➤ High efficiency of gene transfer</td>
<td></td>
</tr>
</tbody>
</table>

efficacy of gene therapy has led to over 600 clinical phase I and II trials including more than 3500 patients with monogenic diseases, cancer, and infectious diseases (www.wiley.co.uk). Although no gene therapy using any type of vector has yet been approved for clinical use, evidence for therapeutic efficacy is suggested by several reports.\(^\text{15-19}\) The first clinical gene therapy trial in patients with severe Crohn’s disease of the rectum is ongoing in which patients receive subcutaneous and submucosal injections of IL-10 and IL-4 cDNA complexed to cationic lipids in the rectal and perianal region.\(^\text{20-22}\)
Introduction

Outline of the thesis

The main purpose of this thesis was to develop a new maintenance treatment for Crohn's disease, based on the principle of regulatory T cells generated by ex vivo transduction. In the past years there has been a shift from the use of non-specific anti-inflammatory agents such as steroids to approaches that intervene specifically in the intestinal inflammatory cascade. Among others, rIL-10 therapy was introduced as a potential new anti-inflammatory therapy. Although systemic administration of rIL-10 was safe and well-tolerated in patients with Crohn's disease, higher doses were associated with an increased incidence of side effects and decreased efficacy (bell-shaped dose response curve).

In chapter 2 we provide an explanation for the lack of efficacy of high doses of rIL-10 in the treatment of Crohn's disease. Our data indicate that high dose rIL-10 (e.g. subcutaneous 20 μg/kg/day) induced the production of the pro-inflammatory cytokine IFN-γ. These findings together with the other limitations of systemic cytokine therapy (short half-life and limited mucosal bioavailability of rIL-10) provided the rationale to study alternative ways of delivering immunoregulatory genes.

In chapter 3 we review the different strategies for gene therapy in the treatment of gastro-intestinal inflammation. Adenoviral delivery of IL-10 would have the advantage of inducing a more sustained expression of IL-10 as compared with administration of rIL-10.

In chapter 4 we studied the potential therapeutic use of an adenoviral vector containing the IL-10 gene in experimental colitis. However, using adenoviral vectors the expression of the transgene remains transient (days to weeks). Retroviral vectors have the potential to induce long-term transgene expression (months to years).

In chapter 5 we employed an ex vivo approach to transfer IL-10 using a retroviral vector to human T cells and performed phenotypic and functional analysis in vivo of the genetic engineered T cells.

In chapter 6 we studied cell migration to the intestine after intravenous cell transfer into mice. We describe the use of animal pinhole single photon emission computed tomography (SPECT), a technique for temporal and spatial imaging of the lymphocyte
Chapter 1

homing process in experimental colitis. Lastly, to obtain the proof of principle for the use of genetic engineered retroviral T cells overexpressing IL-10, in chapter 7 we investigated the efficacy of this approach in offering protection against experimental colitis.

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Introduction


Chapter I


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Introduction


Chapter I


Chapter I


Introduction


Chapter 1


Chapter 1


Introduction


Chapter 1


Introduction


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Chapter I


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