Chapter 8

Summary and Conclusions
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Summary

Crohn's disease is a chronic inflammation of the gastrointestinal tract, characterized by an uncontrolled immune response of intestinal CD4+ T cells to enteric bacteria in genetically susceptible individuals. There is a need for better maintenance therapies for Crohn's disease and the ability to therapeutically intervene has increased as a result of the improved understanding of the pathogenesis of Crohn's disease.

In search for a new maintenance therapy, the immuno-regulatory cytokine interleukin 10 (IL-10) was identified as a promising candidate. IL-10 plays a central role in controlling the homeostasis in the intestinal mucosa, which is best exemplified by IL-10 gene deficient mice that develop chronic colitis. The therapeutic efficacy of recombinant (r)IL-10 was studied both in animal models and in patients with Crohn's disease. Although systemic rIL-10 therapy is safe and well tolerated in patients, the therapeutic benefit of this therapy is modest compared with placebo. Explanations for the limited efficacy include the short half-life of rIL-10 and the delivery of insufficient amounts of rIL-10 to the intestine. Moreover, the systemic administration of rIL-10 is hampered by an increased incidence of side effects and decreased efficacy at higher doses (bell-shaped dose response curve). The use of IL-10 gene transfer to achieve local and sustained expression of IL-10 in the intestine could circumvent the limitations of systemic rIL-10 administration.

The main purpose of this thesis was to develop a new maintenance treatment for Crohn's disease based on IL-10 gene therapy.

In chapter 1 we summarize the current views on the etiology and treatment of Crohn's disease. Furthermore, the function of the intestinal immune system is discussed. In Crohn's disease, the regulatory mechanisms involved in the prevention of chronic inflammation do not function properly, ultimately leading to chronic intestinal inflammation. IL-10 plays an important role in preventing and terminating the mucosal immune response and IL-10 gene transfer to the intestine seems a promising approach for maintenance of remissions in Crohn's disease.

In chapter 2 we investigated the effect of systemic rIL-10 administration in Crohn's disease patients on pro-inflammatory cytokine production to obtain an explanation for
the bell-shaped dose response curve. In two placebo-controlled double blind studies, we analyzed cytokine production in patients with chronic active (CACD) and mild to moderate (MCD) Crohn's disease treated with placebo or subcutaneous rIL-10 daily for 28 consecutive days. In CACD patients serum concentrations of neopterin (a pyrazonopyrimidino derivate) and nitrite/nitrate were measured, all induced by interferon γ (IFN-γ). In ex vivo lipopolysaccharide (LPS)- or phytohaemagglutinin (PHA)-stimulated whole blood cells of MCD patients, we measured IFN-γ and tumour necrosis factor α (TNF-α) release. The IFN-γ-inducible molecule neopterin in CACD patients was significantly increased after treatment with the highest dose of 20 µg rIL-10/kg body weight compared with pretreatment levels. In addition, the PHA-induced release of IFN-γ, but not TNF-α in MCD patients was significantly increased after treatment with the highest dose of 20 µg rIL-10/kg body weight. These data indicate that rIL-10 at high dose (20 µg/kg) increases production of IFN-γ by peripheral blood lymphocytes. Hence, the use of high dose, systemically administered rIL-10 is limited by its lymphocyte-activating and pro-inflammatory effects.

In chapter 3 we review the main features of gene therapy, focussing on the treatment of gastro-intestinal inflammation and retroviral gene transfer. We discuss the basic requirements for gene therapy, including the potential therapeutic genes (transgenes), different vectors (that deliver transgenes) and delivery routes.

In chapter 4 we report on a study that was designed to investigate the therapeutic efficacy of an adenoviral vector containing the IL-10 gene (AdvmuIL-10) in the trinitrobenzene sulfonic acid (TNBS) mouse model of experimental colitis. AdvmuIL-10 was administered intravenously prior to induction of TNBS colitis in mice and the therapeutic efficacy of AdvmuIL-10 was compared with a control virus (an empty cassette virus, Adv0) and with phosphate buffered saline (PBS). AdvmuIL-10 treatment prevented severe body weight loss, and reduced the systemic acute phase response and pro-inflammatory cytokine concentrations in the stool. In addition, the histological scores of intestinal inflammation were significantly lower after treatment with AdvmuIL-10 compared with Adv0- or PBS treatment of TNBS mice. Thus, systemic administration of adenovirus expressing IL-10 reduced parameters of TNBS colitis underscorin the therapeutic potential of IL-10 gene therapy in intestinal inflammation. Adenoviral
transgene expression is transient, because the virus remains episomal and is not incorporated in the host cell genome. We hypothesized that it would be necessary to achieve long-term IL-10 expression because Crohn's disease cannot be cured and often relapses after months to years.

In chapter 5 we describe a study in which the retroviral Moloney Murine Leukemia Virus (MMLV)-derived vector was used. The MMLV vector has the potential to induce long-term transgene expression and is able to infect (=transduce) preselected target cells \textit{ex vivo}. Human peripheral blood mononuclear cells (PBMCs) were transduced with a retroviral vector containing the IL-10 and the green fluorescent protein (GFP) marker gene or a control vector containing the GFP gene only. Transduced CD4^{+} cells were sorted on the basis of GFP fluorescence and maintained in culture for phenotypic and functional analysis. IL-10-GFP CD4^{+} cells responded to CD3/CD28 stimulation with a 6-fold increase in IL-10 production during more than four months. The IL-10 produced was biologically active as evidenced by the following findings: 1) decreased proliferation of IL-10-GFP CD4^{+} cells, 2) decreased expression of major histocompatibility complex class II on the IL-10-GFP CD4^{+} cells, 3) decreased proliferation of autologous responder cells and 4) decreased production of the pro-inflammatory cytokine IL-12 by dendritic cells. The majority of transduced CD4^{+} cells had a gut-homing potential, since they expressed the mucosal adhesion molecule α4β7, and displayed efficient binding to the mucosal addressin MAdCAM-1-expressing cells \textit{in vitro}. These data indicate that transduction of peripheral blood CD4^{+} lymphocytes with IL-10 results in an immune-regulatory function of these cells.

In chapter 6 we applied the recently developed and validated animal pinhole single photon emission computed tomography (SPECT) technique for \textit{in vivo} visualization of radioactive labelled lymphocyte migration to the intestine in mice. To study the migration pattern of lymphocytes after transfer, we isolated lymphocytes from the spleens of healthy mice and mice with TNBS colitis (sensitized lymphocytes). Subsequently, the cells were radioactively labelled with \textsuperscript{111}Indium-oxinate and injected intravenously into control mice or mice with TNBS colitis. On SPECT analysis, the radioactive colon uptake was most evident in mice with TNBS colitis that received sensitized lymphocytes 48 \textit{h} after cell transfer. Our findings were confirmed by standard planar scintigraphy. The
sensitized In labelled lymphocytes retained their functionality and even exacerbated colitis compared with non-sensitized lymphocytes. The colon radioactivity uptake correlated well with parameters of colitis (colon weight and histological score of intestinal inflammation). Administration of an anti-α4 antibody (blocks α4-mediated adhesion) decreased radioactivity uptake in the colon compared with a control antibody. Together, these findings demonstrate that SPECT can be applied for temporal and spatial analysis of the lymphocyte homing process in experimental colitis, and for assessment of efficacy of new therapeutic strategies that intervene with lymphocyte migration.

In chapter 7 we show that transfer of engineered murine lymphocytes that express IL-10 offer protection against experimental colitis. Spleen-derived CD4 cells were transduced using the MMIAV vector containing the human IL-10 and GFP gene. Transduced cells were injected into severe combined immunodeficient (SCID) mice, before induction of chronic colitis by transfer of a pathogenic population of CD45RB<sup>high</sup> CD4<sup>+</sup> cells, and into BALB/c mice before induction of acute TNBS colitis. IL-10-GFP CD4<sup>+</sup> cells prevented CD45RB<sup>high</sup> induced colitis effectively, whereas no therapeutic effect was observed after injection of non-transduced CD4<sup>+</sup> cells. In addition, IL-10-GFP CD45RB<sup>high</sup> CD4<sup>+</sup> cells lost the capacity to induce colitis when compared with non-transduced CD45RB<sup>high</sup> CD4<sup>+</sup> cells. By contrast, no therapeutic benefit was observed in TNBS-induced colitis. From this study we conclude that primary murine CD4<sup>+</sup> cells, engineered to express IL-10 by retroviral transduction, act as regulatory cells in CD45RB<sup>high</sup> CD4<sup>+</sup> cell-induced colitis.

Conclusions and future perspectives

The studies presented in this thesis provide a rationale for the use of IL-10 gene therapy in Crohn’s disease and demonstrate the therapeutic principle of this approach in experimental colitis. In vivo intravenous administration of an adenoviral vector expressing the IL-10 gene prevented severe colitis in mice. Although the duration of IL-10 expression after adenoviral gene transfer will be longer than after administration of rIL-10, expression of adenoviral IL-10 is transient and systemic side effects may occur
after intravenous administration. Using the *ex vivo* approach it was possible to transduce human CD4+ cells with a retroviral vector and achieve long-term expression of bioactive IL-10 *in vitro*. Murine IL-10 transduced CD4+ cells prevented the development of experimental colitis *in vivo*.

Clinical experience published so far indicated only a modest therapeutic effect of systemic IL-10 therapy. However, our results have shed new light on the possibilities for IL-10-based therapy in Crohn's disease. Moreover, IL-10-based gene therapy has therapeutic potential beyond Crohn’s disease and can be applied in a wide variety of T cell mediated inflammatory diseases (e.g. rheumatoid arthritis, multiple sclerosis, psoriasis, allergy, transplantation rejection). Besides IL-10, a wide range of therapeutic transgenes can be explored. Of course, the development of IL-10 gene therapy has only just started and the pre-clinical evidence needs to be translated to the clinical setting.

We consider the following points relevant for further development of IL-10 gene therapy: for maintenance treatment of Crohn’s disease long-term transgene expression is desirable. The duration of expression is determined by the vector type, the promoter that regulates transcription of the transgene, and the life span of the target cells. Furthermore, the regulation of transgene expression, the specificity of the treatment, the level of IL-10 expression, the number of cells that needs to express IL-10 and the safety of the gene therapy should be considered.

For long-term transgene expression, an adeno-associated viral (AAV) vector can be further explored for intraluminal, *in vivo* administration. AAV provides a more sustained transgene expression when compared with the adenoviral vector studied here because it integrates in the host cell genome. Alternatively, using an *ex vivo* approach, lentiviral vectors are attractive vectors. Lentiviral vectors integrate in the host cell genome of non-dividing cells, eliminating the need to stimulate the target cells prior to transduction and reducing the period of cell culture.

The duration of transgene expression *in vivo* is unknown for most vectors and not only depends on vector characteristics, but also on the activity of the promoter used. Transcriptional silencing of retroviral promoters does occur *in vivo* but vectors can be modified or different promoters can be used to prevent this phenomenon. The lifespan of the target cells is also a determining factor for the transgene expression. When CD4+
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cells are used, long-term gene expression can be expected since CD4* cells, in particular memory CD4* cells, potentially have a long life span.

Long-term expression of transgenes needs to be regulated, and for this purpose tissue-specific or activation-dependent promoters can be incorporated in the viral vector. For example, the 'liver' fatty acid binding protein promoter expressed by the continuously regenerating epithelium may be used to obtain local transgene expression within the intestine, or the IL-2 promoter can be inserted for transgene expression upon T cell activation. In the work presented in this thesis, we have shown the natural inducibility of the retroviral MMLV promoter (LTR) in response to stimulation of the T cell receptor of transduced CD4* cells. Furthermore, to obtain a "failsafe" control mechanism, a suicide gene that induces cell death upon exposure to specific drugs can be incorporated in the vector, for example the CD20 surface marker gene that is recognized by the humanized anti-CD20 monoclonal antibody rituximab, or the gene for the herpes simplex virus-1 thymidine kinase that combined with ganciclovir induces cell death.

To prevent systemic side effects using the ex vivo approach, the IL-10 producing cells that migrate to the intestine could be sorted before adoptive transfer. The IL-10 transduced cells may be selected employing an isolation procedure for IL-10 secreting cells. Alternatively, a bicistronic vector can be used containing both the IL-10 gene and a surface marker, such as CD20 or the low affinity nerve growth factor gene. Transduced cells expressing these surface markers can be selected with antibodies. Subsequently, local IL-10 expression in the intestine may be accomplished by sorting the transduced cells -before adoptive transfer- for expression of the gut-homing adhesion molecule α4β7.

The concentration of IL-10 in the intestine and the number of cells that needs to be transduced for a therapeutic effect is unknown. We have shown that systemic administration of high dose rIL-10 induces pro-inflammatory effects, indicating the therapeutic window of IL-10. We hypothesize that relatively low local IL-10 concentrations are needed to control mucosal inflammation based on the following findings: First, a study by Steidler et al showed that experimental colitis was prevented by a much lower concentration of IL-10, produced locally in the intestine by genetic modified bacteria, than obtained by systemic rIL-10 administration. Second, in healthy volunteers and patients with Crohn's disease about 0.5-2% of PBMCs secrete IL-10 (our
unpublished data, not presented in this thesis), and this low percentage is sufficient to maintain homeostasis in healthy persons. Finally, it has been shown that in experimental arthritis a small number of transduced cells overexpressing IL-10 can ameliorate joint inflammation.

Safety issues such as insertional mutagenesis, toxicity, immune response against the vectors and long-term effects will have to be addressed in pre-clinical and clinical studies. However, a large number of patients has been treated with retroviral vectors, and this has not yet led to an increased incidence of lymphoma’s or other malignancies compared with the non-treated population.

Clearly, we are only starting to grasp the therapeutic potential of the approach presented in this thesis. Nonetheless, the preliminary experiments reported in this thesis indicate that altering the functional properties of T cells by \textit{ex vivo} gene therapy is a promising approach for long-term control of the mucosal immune response in patients with Crohn’s disease. Our results provide a strong rationale for further clinical development of the chosen approach.

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