Interleukin 10 gene therapy for Crohn's disease
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Prevention of colitis by interleukin 10 transduced T lymphocytes in the SCID mice transfer model

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resubmitted with revisions to Gastroenterology
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Abstract

Background & Aims: Regulatory CD4⁺ cells secreting the anti-inflammatory cytokine IL-10 play a key role in maintaining the immune balance in the intestinal mucosa. In this study we engineered primary CD4⁺ cells to express IL-10, and investigated the efficacy of this approach in offering protection against experimental colitis.

Methods: Spleen-derived CD4⁺ cells were transduced using a retroviral (MMLV) vector to simultaneously express IL-10 and green fluorescent protein (GFP). The therapeutic benefit of CD4⁺ cells transduced with IL-10-GFP was studied in experimental chronic colitis, induced by transfer of CD45RB⁺⁺ CD4⁺ cells to SCID mice, and in acute TNBS-induced colitis.

Results: Transferred engineered GFP fluorescent cells were detected for at least 15 weeks in peripheral blood, spleens, colon, and lymph nodes draining the intestine of recipient SCID mice. IL-10-GFP CD4⁺ cells prevented CD45RB⁺⁺ CD4⁺ cell-induced transfer colitis effectively, whereas no effect was observed after transfer of non-transduced CD4⁺ cells. IL-10-GFP CD45RB⁺⁺ CD4⁺ cells lost the capacity to induce colitis. By contrast, no therapeutic benefit was observed in TNBS-induced colitis.

Conclusions: Primary murine CD4⁺ cells that were engineered to express IL-10 by retroviral transduction act as regulatory cells in CD45RB⁺⁺ CD4⁺ cell-induced transfer colitis. This approach may induce long-term maintenance of mucosal immune homeostasis in Crohn’s disease.
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Introduction

Antigen-dependent CD4+ cell proliferation and enhanced secretion of pro-inflammatory cytokines are pivotal in the initiation of mucosal inflammation in both humans and animal models. Recent therapeutic strategies for Crohn's disease have focused on targeting pro-inflammatory cytokines and their receptors. It has become apparent that, besides having anti-inflammatory effects, several of these therapies also alter T-cell function. For example, the clinical benefit of the anti-tumour necrosis factor α (TNF-α) antibody infliximab in Crohn's disease seems to be mediated, at least partly, by induction of apoptosis of mucosal lymphocytes, and the efficacy of blockade of IL-12 or IL-6 in animal models is also related to apoptosis induction. Hence, inhibition of T cell activation can cause remissions in (animal models of) Crohn's disease. Although blockade of TNF-α results in a therapeutic response in the majority of patients with active Crohn's disease, in most patients remissions are transient. The mucosal immune system seems therefore unable to control inflammation in patients with Crohn's disease, even after induction of a complete remission.

Regulatory CD4+ cells play a dominant role in controlling mucosal inflammation in part by secretion of anti-inflammatory cytokines such as interleukin-10 (IL-10). Unfortunately, systemic treatment of patients with Crohn's disease with recombinant (r)IL-10 has resulted in only a modest therapeutic benefit, presumably due to limited mucosal bioavailability and pro-inflammatory effects of high-dose IL-10. In addition, it is likely that IL-10 functions as a regulatory cytokine in the context of intimate cell-cell interactions, rather than as a circulating cytokine. Clinical application of regulatory CD4+ cells is precluded by the low proliferation rate of these cells and the high cost of the rIL-10 required for their generation in vitro. An alternative approach is the use of T cells that are genetically engineered to express regulatory cytokines.

The concept of employing T cells as vehicles for delivering regulatory cytokines has been investigated in several Th1-biased animal models of human disease. An anti-myelin basic protein T cell clone transduced with transforming growth factor β (TGF-β) protected mice against experimental autoimmune encephalomyelitis induced by immunization with either myelin basic protein or proteolipid protein. In experimental
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arthritis, antigen specific T cells transduced with IL-10 or IL-4 ameliorated the disease without impairing systemic immune responses to the antigen.

In this study we have explored the therapeutic effect of T cells transduced \textit{ex vivo} with IL-10 in two murine models of colitis. For IL-10 gene transfer into T cells retroviral techniques were employed, since they are efficient and non-cytotoxic, and result in long-term gene expression. Our results indicate that IL-10 transduced CD4+ cells prevent CD45RB\textsuperscript{high} induced transfer colitis successfully, and that IL-10 transduced CD45RB\textsuperscript{high} CD4+ cells lost the capacity to induce colitis. By contrast, no therapeutic benefit was observed in TNBS-induced colitis. These data indicate that local delivery of therapeutic proteins via CD4+ T cells may be a promising strategy for controlling the failing mucosal immune balance in Crohn's disease.

Materials and Methods

Mice

BALB/c and C.B.-17 SCID mice were purchased from Charles River (Charles River, Someren, the Netherlands) and maintained in filter-top cages under specific-pathogen free conditions at our animal care facility. All experiments were approved by the animal welfare committee. Mice were used at 7-10 weeks of age.

Production of replication-defective retrovirus

The LZRS\textsuperscript{PMN-IRE\textsuperscript{S}-GFP} retroviral plasmid (referred to as GFP) was constructed as described previously. Briefly, GFP complementary DNA (cDNA) (Clontech, Palo Alto, CA) was cloned downstream of an internal ribosome entry site (IRE\textsuperscript{S}) in the LZRS retroviral vector (kindly provided by G. Nolan) and expressed from the retroviral long terminal repeat of the Moloney Murine Leukemia Virus (MMLV). A 536-bp PCR fragment containing the human IL-10 cDNA\textsuperscript{22} was amplified from human cDNA with primers containing a BamHI (fw: GGATCCACCATGCAAGCTCAGCATCACTGCTGT) and a Xho I (rv: CTCGAGTGATTTCTCGTATCTCCATGTCATGT) restriction
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The fragment was sequenced and cloned in the BamHI/XhoI sites upstream of the IRES in LZRSpBMN-IRES-GFP. The final bicistronic retroviral reporter construct was termed LZRSpBMN-II-10-IRES-GFP and referred to as IL-10-GFP.

Transfection of an amphotropic producer cell line was performed as described previously. Viral supernatants were used to infect the ecotropic 293T Phoenix packaging cells (kindly provided by G. Nolan) for 16 hours (h) in the presence of 10 μg/ml DEAE (Sigma, St Louis, MI). Single infected Phoenix cells were sorted by a FACS Vantage Cell Sorter (Becton Dickinson, Mountain View, CA) using an automatic cell deposition unit (Becton Dickinson), and virus-containing supernatants were generated with titres of 10^5-10^6 IU/ml.

Infection of splenocytes

BALB/c splenocytes were isolated using filter cell strainers (Becton Dickinson) and red cells were lysed. Splenocytes (3-5x10^6/well) were seeded in 24 well plates (Costar Europe Ltd, Badhoevedorp, The Netherlands) in Iscove's modified Dulbecco's medium (Biowhittaker) supplemented with 10% FCS (Biowhittaker), 50 μM 2-ME (Merck, Darmstadt, Germany), 1% penicillin-streptomycin-glutamine solution (GibcoBRL, Grand Island, NY) and 20 U/ml of rIL-2 (Chiron, Amsterdam, The Netherlands). Cells were activated for 24 h with immobilized anti-αCD3 (1:30 concentration, clone 145-2C11) and soluble αCD28 monoclonal antibodies (mAb) (1:1000 concentration, Pharmingen, San Diego, CA) under standard conditions (37°C, 5% CO₂). Retrovirus-containing supernatants were added for overnight incubation. Medium was exchanged with supplemented Iscove's medium and αCD28 mAb (1:3000 concentration, Pharmingen) for an additional 48 h.

Splenocytes were harvested and stained with cyochrome-conjugated αCD4 mAb (Coulter-Immunotech, Marseille, France) and routinely consisted of 42 ± 5% CD4⁺ cells. Cells were either used directly for injection into BALB/c mice or, in separate experiments, sorted into subsets of CD4 and GFP expressing cells using a FACS Vantage Cell Sorter (Becton Dickinson). Sorted subsets were reanalyzed following the initial collection to confirm fraction purity, which was routinely >90%. In a second set of experiments, splenocytes were also stained using phycoerythrin (PE)-conjugated
### Table 1 Experimental set-up transfer IL-10 CD4+ cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Cells injected</th>
<th>Number of cells x 10^5 (number of mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phenotype</td>
<td>exp 1</td>
</tr>
<tr>
<td>1</td>
<td>CD45RB^{hi} CD4+ cells</td>
<td>1.2 (n=7)</td>
</tr>
<tr>
<td>2</td>
<td>CD45RB^{hi} CD4+ cells + CD45RB^{lo} CD4+ cells</td>
<td>1.2 +</td>
</tr>
<tr>
<td>3</td>
<td>CD45RB^{hi} CD4+ cells + IL-10-GFP CD4+ cells</td>
<td>1.2 +</td>
</tr>
<tr>
<td>4</td>
<td>CD45RB^{hi} CD4+ cells + control CD4+ cells</td>
<td>1.2 +</td>
</tr>
</tbody>
</table>

CD45RB (Coulter-Immunotech) and sorted into subsets of CD45RB^{hi} CD4 and GFP expressing cells. Upon reanalysis, the sorted cells were > 90% pure. A small fraction of the sorted cells (1x10^5/well) was used for analysis of cytokine production and the remaining cells were injected intraperitoneally in recipient mice (see below).

### Induction of CD45RB^{hi} transfer colitis and treatment protocol

Chronic CD45RB^{hi} transfer colitis was induced as previously described. Briefly, BALB/c splenocytes were first enriched for CD4+ cells by red cell lysis and negative selection using the following rat anti-mouse mAbs: B220 (clone RA3-6B2), Mac-1 (clone M1/70), and CD8α (clone 53-6.7) (gift from Dr. R. Mebius, Vrije Universiteit Medical Center, Amsterdam, The Netherlands). MAb-stained cells were removed in a magnetic field using sheep anti-rat IgG coated magnetic beads (Dynal, Hamburg, Germany). The resulting CD4+ cells were stained with cyochrome (Cy)-conjugated CD4 and fluorescein isothiocyanate (FITC)-conjugated CD45RB (both Pharmingen) mAbs. Subpopulations of CD4+ cells were generated by two colour sorting on the FACS sorter (Becton Dickinson). Populations were > 95% pure upon reanalysis.

C.B-17 SCID mice received intraperitoneal injections of sorted CD4+ cell subpopulations in PBS. To induce colitis, CD45RB^{hi} CD4+ cells (1-4x10^5) were transferred to four groups of mice in a first set of experiments (table 1). The mice
Table 2: Experimental set-up for IL-10 CD45RB<sup>hi</sup> cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Cells injected</th>
<th>Phenotype</th>
<th>Number of cells x 10&lt;sup&gt;6&lt;/sup&gt; (number of mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CD45RB&lt;sup&gt;hi&lt;/sup&gt; CD4&lt;sup&gt;+&lt;/sup&gt; cells</td>
<td>exp 1: n=7</td>
<td>2 (n=7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>exp 2: n=4</td>
<td>2 (n=4)</td>
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<tr>
<td></td>
<td></td>
<td>exp 3: n=3</td>
<td>2 (n=3)</td>
</tr>
<tr>
<td>2</td>
<td>IL-10-GFP CD45RB&lt;sup&gt;hi&lt;/sup&gt; CD4&lt;sup&gt;+&lt;/sup&gt; cells</td>
<td>exp 1: n=5</td>
<td>2 (n=5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>exp 2: n=4</td>
<td>2 (n=4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>exp 3: n=3</td>
<td>2 (n=3)</td>
</tr>
</tbody>
</table>

received CD45RB<sup>hi</sup> CD4<sup>+</sup> cells alone (Group 1) or in combination with CD45RB<sup>lo</sup> CD4<sup>+</sup> cells (Group 2), sorted IL-10-GFP CD4<sup>+</sup> cells (Group 3) or control CD4<sup>+</sup> cells (Group 4). In a second set of experiments, SCID mice received either non-transduced control CD45RB<sup>hi</sup> CD4<sup>+</sup> cells or IL-10-GFP transduced CD45RB<sup>hi</sup> CD4<sup>+</sup> cells (table 2).

**Induction of TNBS colitis and treatment protocol**

Acute colitis was induced in BALB/c mice by rectal administration of two doses (separated by a 7 day interval, i.e. on days 0 and 7) of 2 mg TNBS (Sigma Chemical Co., St. Louis, MO, USA) in 40% ethanol (Merck, Darmstadt, Germany), as described previously. On the day of the first TNBS administration, the mice received an intravenous injection of either non-transduced control splenocytes or non-sorted IL-10-GFP transduced splenocytes. Nine days after the first TNBS administration the mice were sacrificed.

**Assessment of inflammation**

Mice with chronic colitis (transfer model) were weighed twice a week and mice with acute colitis (TNBS model) were weighed daily. Body weight loss was determined by percentage of weight loss from baseline body weight. Peripheral blood was drawn at different time points by retro-orbital sinus puncture for plasma IL-10 measurement and FACScan analysis of GFP expression. At necropsy, colons were removed through a midline incision and opened longitudinally. The wet weight of the distal 6 cm was used as an index of disease-related intestinal wall thickening. Subsequently, the colons were
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longitudinally divided into two parts: one for histology, the other for cytokine assay. RNA isolation or detection of GFP expressing cells. Intestinal cells and colon homogenates were prepared as described in an earlier study. Cell suspensions of the spleens, the caudal and the mesenteric lymph nodes were isolated and, if cell numbers were sufficient, plated (1x10^5 cells/well) on 96-well round bottom plates (Costar) in a final volume of 200 µl in the presence of αCD3/CD28 mAbs for assessment of cytokine production. The remaining cells were resuspended in FACS buffer for detection of GFP expression. Culture supernatants were collected from 4 wells after 48 h, pooled and stored at -20°C until use.

Cytokine analysis

Cytokine concentrations (IL-2, IL-4, IL-5, TNF-α and IFN-γ) in cell culture supernatants derived from spleen and lymph nodes were measured by a cytometric bead assay (CBA, Becton Dickinson) according to manufacturer's instructions. Briefly, a mixture of cytokine capture beads that have discrete fluorescence intensity characteristics, was added to the supernatants and cytokine standards. Next, samples were incubated at RT with a Th1/Th2 PE-conjugated detection reagent, containing α-murine IL-2, IL-4, IL-5, TNF-α and IFN-γ Abs. After 2 h, samples were washed in a wash buffer and analyzed on a FACS Calibur using CBA software (both Becton Dickinson). Expression of human IL-10 was analyzed in plasma samples, culture supernatants of spleens, caudal and mesenteric lymph nodes and in colon homogenates by ELISA (CLB, Amsterdam, The Netherlands). A TNF-α ELISA was performed on the colon homogenates (R&D Systems, Abingdon, UK).

Analysis of GFP expression

Cells from peripheral blood, colon, spleen and lymph nodes were incubated for 20 min on ice with fluorochrome-conjugated CD4-Cy or an isotype control mAb (both Pharmingen) and analyzed using a FACS Calibur in conjunction with FACScan software (both Becton Dickinson). Gates were set to exclusively detect viable lymphocytes and negative green fluorescence was set at less than 1%, using cells from a mouse that had received control CD4<sup>+</sup> cells.
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Histology
The longitudinally divided colons were rolled up and fixed in 4° buffered formalin. Fixed tissues were embedded in paraffin, and 4-6 μm sections were stained with haematoxylin and eosin for histological grading. An experienced pathologist blinded to treatment allocation scored all sections. In the transfer model inflammation was scored on a scale of 0-4, representing no inflammation to severe inflammation. A different scoring system was used for the TNBS colitis model using the following parameters 1) percentage of colon involved, 2) fibrosis, 3) edema, 4) erosions and ulcers, 5) crypt loss, 6) infiltration of mononuclear cells and 7) polymorphonuclear cells as described previously. The total score ranges from 0 (normal colon) to a maximum of 20 points (most severe inflammation).

RT-PCR for IL-10-GFP
Total RNA was isolated from colon homogenates using TRIZOL (Gibco BRL) and treated with RNAase-free DNAase (GibcoBRL). First strand cDNA synthesis was carried out with 2-5 μg total RNA, 0.5 mM dNTPs, 250 ng random primers, 10 μM DTT, 40U Ribonuclease inhibitor and 200U of MMLV reverse transcriptase (RT) in a final volume of 20 μl, with buffer and incubations according to the instructions of GibcoBRL. Separate reaction tubes without reverse transcriptase were used as control of DNA contamination. PCR was performed in a thermocycler Gene AMP\textsuperscript{8} PCR System 9700 (Perkin Elmer, Norwalk, CT, USA) using 2-4 μl of cDNA template, 0.2 mM dNTPs, 0.2 μM of each primer (IL-10fw 5'CTAACGTTCTGGCCGAAGC3'; GEPrev 5'TCTTGTAGTGCGCGTCC3') and 1U of AmpliTag polymerase (Perkin Elmer Corp., Branchburg, NJ, USA), in a final volume of 25 μl containing 10 mM Tris-HCl pH 9.0, 1.5 mM MgCl\textsubscript{2}, 50 mM KCl and 0.1% Triton X-100. The cycling conditions were as follows: denaturation 94°C for 4 min, then 30 cycles of (94°C, 30 sec; 50°C 30 sec; 72°C 45 sec) and a final extension at 72°C for 5 min. A second PCR using β-actin primers (m\textsubscript{3}actinfw 5'GTCAGAAGGATCGCTATG TG3'; m\textsubscript{3}actinrv 5'GCTCGTGGCCAATAGTGATG3') was performed under the same conditions. The PCR products were separated on a 1% agarose gel and visualized by UV illumination.
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Statistical analysis

Differences between treatment groups were analyzed by the Mann-Whitney U test. Differences between treatment groups in time were tested by analysis of variance (ANOVA) for repeated measures. Results are expressed as median (range) or as mean ± SEM where appropriate. All statistical tests were done using SPSS for Windows (SPSS Inc, Chicago, Illinois, USA). A two-tailed p value of less than 0.05 was considered to represent a significant difference.

Results

Efficient transduction and sorting of murine splenocytes

To generate IL-10 expressing CD4<sup>+</sup> cells, activated murine splenocytes were transduced with the IL-10-GFP retroviral construct. The percentage of GFP-expressing cells (transduction efficiency) in several independent experiments (n=11) was 16 ± 2<sup>%</sup>. After 48 h, viable GFP<sup>-</sup>CD4<sup>+</sup>-expressing splenocytes were sorted using a FACS Vantage flow cytometer, while GFP<sup>-</sup>CD4<sup>+</sup> cells served as negative controls, resulting in populations consisting of 89 ± 2<sup>%</sup> (GFP<sup>-</sup>CD4<sup>+</sup>) and 97 ± 1<sup>%</sup> (GFP<sup>-</sup>CD4<sup>+</sup>) cells respectively. The culture supernatants from activated and resting IL-10-GFP CD4<sup>+</sup> cells (1x10<sup>6</sup>/ml) contained up to 5700 and 178 pg IL-10/ml/48 h respectively. By contrast, the supernatants of non-transduced cells did not contain detectable levels of IL-10. As we previously observed in human CD4<sup>+</sup> cells, the transduction procedure did not significantly alter CD45RB expression (memory marker) or cytokine production (IL-2, IL-4, IL-5, IFN-<gamma>, and TNF-<alpha>) in the absence or presence of αCD3/CD28 activation (data not shown).

Long-term survival of IL-10-GFP cells

Colitis was induced in SCID mice by the transfer of CD45RB<sup>hIGH</sup> CD4<sup>+</sup> cells. SCID mice received CD45RB<sup>hIGH</sup> CD4<sup>+</sup> cells alone (Group 1) or in combination with a single administration of CD45RB<sup>hi</sup> CD4<sup>+</sup> cells (Group 2), IL-10-GFP CD4<sup>+</sup> cells (Group 3) or control non-transduced CD4<sup>+</sup> cells (Group 4) (see table 1).
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Figure 1 Survival of GFP expressing cells in peripheral blood of SCID mice reconstituted with CD45RB\textsuperscript{high} CD4\textsuperscript{+} cells

FACS analysis of peripheral blood was performed at 2, 7, 13 and 15 weeks after transfer of IL-10-GFP (upper panel) or control CD4\textsuperscript{+} cells (lower panel). The y-axis represents CD4 expression and the x-axis represents GFP fluorescence of gated viable lymphocytes. Representative images of 1 out of 12 mice are shown. Mean percentages of GFP\textsuperscript{+} cells in the CD4\textsuperscript{+} population are indicated.

To study the survival of IL-10-GFP CD4\textsuperscript{+} cells, we analyzed the GFP expression of peripheral blood cells in the recipient mice by FACS at different times after cotransfer of IL-10-GFP and CD45RB\textsuperscript{high} CD4\textsuperscript{+} cells. As shown in figure 1, GFP\textsuperscript{+}CD4\textsuperscript{+} cells were detected at all time points tested (2, 7, 13 and 15 weeks) in all the mice (n=12), and after 15 weeks constituted a substantial proportion (8.6 ± 1.0\%) of the CD4\textsuperscript{+} population. Virtually no (auto)fluorescent cells were detected following transfer of control CD4\textsuperscript{+} cells (0.9 ± 0.3\%, n=12). In all the mice tested (n=6 from each group) circulating levels of IL-10 were below the detection limit 2 weeks after transfer and upon sacrifice. Transfer of IL-10-GFP CD4\textsuperscript{+} cells resulted in GFP\textsuperscript{+} expressing cells in the spleen (11.2 ± 0.7\% of CD4\textsuperscript{+}cells) and caudal lymph node (10.4 ± 1.2\% of CD4\textsuperscript{+}cells), which drains the large intestine. Less than 1\% CD4\textsuperscript{+} cells displayed (auto)fluorescence in the same organs after transfer of control CD4\textsuperscript{+} cells (n=12). Hence, the IL-10-GFP transduced CD4\textsuperscript{+} cells persisted \textit{in vivo}.
Figure 2 IL-10 transduced CD4^+ cells reduce wasting and increase of colon weight

SCID mice received CD45RB^hi CD4^+ cells alone or in combination with other subpopulations as indicated. A) Body weights, measured at the end of the experiments, are expressed as a percentage of initial body weight. IL-10-GFP CD4^+ treated mice had significantly higher body weights than untreated (p=0.013) or control CD4^+ cell treated mice (p=0.031). B) The weight of the last 6 cm of the colon was determined upon sacrifice. IL-10-GFP CD4^+ treated mice had significantly lower colon weights than untreated (p=0.034) or control CD4^+ cell treated mice (p=0.007). Each symbol represents an individual mouse. Bar indicates median weight for each group. Data are pooled from two separate experiments.

IL-10 transduced CD4^+ cells prevent transfer colitis

To determine whether the IL-10-GFP CD4^+ cells were able to prevent colitis induced by transfer of CD45RB^hi CD4^+ cells to SCID mice, we performed the experiments summarized in table 1. In these experiments, the interval between transfer of CD45RB^hi cells and cotransfer of IL-10-GFP transduced cells ranged from 4 to 14 days. The results of the experiments were identical, and the data were therefore pooled. After 15-18 weeks, mice transferred with CD45RB^hi CD4^+ cells alone (Group 1) or cotransferred with control CD4^+ cells (Group 4) developed a hunched appearance and lost weight (figure 2A); the experiment was terminated and all the mice were sacrificed.

As previously described,^{27} transfer of CD45RB^hi CD4^+ cells into SCID mice resulted in severe colitis, with an increase in colon weight (figure 2B) and histological signs of mucosal inflammation (figure 3 and 4).
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Figure 3 Colon of SCID mice after transfer of CD45RB\(^{\text{hi}}\) CD4\(^{+}\) cells alone or in combination with other subpopulations

A) Severe colitis induced by CD45RB\(^{\text{hi}}\) CD4\(^{+}\) cells, characterized by an extensive inflammatory cell infiltrate, epithelial cell hyperplasia, and loss of goblet cells. B) Colon of a mouse treated with CD45RB\(^{\text{lo}}\) CD4\(^{+}\) cells. This picture shows normal colon architecture. C) Colon of a mouse treated with IL-10-GFP CD4\(^{+}\) cells, showing a normal architecture with a small number of leukocytes in the mucosa and a large number of goblet cells in the crypts. D) Colitis in a mouse treated with control CD4\(^{+}\) cells; histological features included crypt hyperplasia, ulceration and crypt abscesses. Hematoxylin and eosin staining. Original magnifications: 33 x.

Mice that were cotreated with CD45RB\(^{\text{lo}}\) CD4\(^{+}\) cells (Group 2), whose protective effect in this model is on record,\(^\text{27}\) did not develop colitis as reflected by normal body weight gain and normal colon weights (figure 2). Treatment with IL-10-GFP CD4\(^{+}\) cells (Group 3) also protected the mice from induction of colitis: by the end of the experiment body weights were significantly higher compared with the untreated (p=0.013) or control CD4\(^{+}\) cell treated mice (p=0.031), and colon weights were significantly lower compared with the untreated (p=0.034) or control CD4\(^{+}\) cell treated mice (p=0.007). By contrast, cotransfer of the non-transduced control CD4\(^{+}\) cells (Group 4) did not offer protection
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CD45RB<sup>+</sup> CD4<sup>+</sup> cells +
Gr 1: no treatment
Gr 2: CD45RB<sup>+</sup> CD4<sup>+</sup>
Gr 3: IL-10-GFP CD4<sup>+</sup>
Gr 4: control CD4<sup>+</sup>

**Figure 4 Prevention of colitis in mice treated with IL-10-GFP CD4<sup>+</sup> cells**

SCID mice received CD45RB<sup>high</sup> CD4<sup>+</sup> cells alone or in combination with other subpopulations as indicated. The extent of mucosal inflammation was examined and graded (see materials and methods). Each symbol represents an individual mouse. Bars indicate mean colitis score for each group and * indicates a significant difference between IL-10-GFP CD4<sup>+</sup> cell treated mice and untreated (p<0.001) or control CD4<sup>+</sup> cell treated mice (p<0.001).

From colitis, and even seemed to aggravate the signs (figure 2). Histological pictures of colons of recipient SCID mice and total histological scores are shown in figures 3 and 4 respectively. Colitis in mice that received CD45RB<sup>high</sup> CD4<sup>+</sup> cells (figure 3A) was characterized by an extensive inflammatory cell infiltrate, marked crypt hyperplasia, and loss of goblet cells (histological score 2.33 ± 0.28). The large intestine of mice, cotransferred with CD45RB<sup>low</sup> CD4<sup>+</sup> cells (figure 3B) showed either no change, or minimal changes consisting of influx of a few leukocytes and slight epithelial hyperplasia (histological score 0.33 ± 0.14). Similarly, in IL-10-GFP CD4<sup>+</sup> cell recipients (figure 3C) either no histological changes were found, or minimal ones consisting of slight crypt hyperplasia and some influx of leukocytes in the mucosa (histological score 0.72 ± 0.19). By comparison, crypt abscesses and ulcerations were the signs of colitis seen in the recipients of control CD4<sup>+</sup> cells (histological score 2.42 ± 0.29) (figure 3D). The histological scores for all groups are shown in figure 4. Colonic inflammation was
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reduced significantly after transfer of IL-10-GFP CD4+ cells compared with the untreated (p<0.001) or control CD4+ cell treated mice (p<0.001).

Transfer of IL-10 CD4+ cells reduces pro-inflammatory cytokine levels

To investigate the mechanism by which IL-10-GFP CD4+ cells influenced the development of colitis, we quantified cytokine production of caudal lymph node and splenic lymphocytes isolated from mice treated with IL-10-GFP or control CD4+ cells. Cells were stimulated with αCD3/CD28 mAb in vitro, and IL-2, IL-4, IL-5, IFN-γ and TNF-α levels in culture supernatants were measured using a CBA. Production of these cytokines by splenocytes was not altered by treatment with IL-10-GFP CD4+ cells (data not shown). By contrast, production of IFN-γ and TNF-α by caudal lymph node cells was lower in the IL-10-GFP CD4+ cell treated mice (n=7 tested) (TNF-α 253 (40-1165) and IFN-γ 814 (40-6279) pg/ml/48 h) than in the control CD4+ cell treated mice (n=5) (TNF-α 924 (120-5000) and IFN-γ 3675 (240-13925) pg/ml/48 h), although no statistical significance was reached. In addition, when colon homogenates were analyzed by ELISA, a reduction in TNF-α (n=6 tested) levels was observed in IL-10-GFP CD4+ cell treated mice compared with control CD4+ cell treated mice (n=6 tested) (101 (46-135) versus 253 (53-397) pg/ml, not significant).

IL-10 levels were below the detection limit (<2.4 pg/ml) in the colon homogenates. However, IL-10 was detected in 4 out of 8 caudal lymph node and 7 out of 12 spleen cell supernatants of mice treated with IL-10-GFP CD4+ cells. By contrast, IL-10 levels were below the detection limit in all caudal lymph node (n=5) and spleen cell (n=11) supernatants of mice treated with control CD4+ cells. These results suggest that the transfer of IL-10 producing CD4+ cells mainly inhibited inflammation in the local mucosal compartment, without interfering with systemic (spleen) immune activation.

IL-10 transduced CD45RBhigh CD4+ T cells are non-pathogenic in SCID mice

An earlier study reports that transfer of CD45RBhigh CD4+ cells isolated from IL-10 transgenic mice does not cause colitis in SCID mice.2a We therefore performed a second set of experiments, in which CD45RBhigh CD4+ cells were sorted into IL-10-GFP
Figure 5 IL-10-GFP transduced \( \text{CD}45\text{RB}^{\text{high}} \text{CD}4^{+} \) cells prevent loss of body weight

SCID mice received equal numbers of control or IL-10-GFP CD45RB\(^{\text{high}} \) CD4\(^{+} \) cells as indicated. Body weights were recorded twice weekly during the entire experiment. The change of weight is expressed as the mean percentage of initial weight per group ± SEM. ANOVA for repeated measures indicated that bodyweights of the two groups differed significantly in time (\( p<0.001 \)). Data are pooled from three separate experiments.

Transduced and non-transduced subsets in order to test for their ability to induce colitis after transfer into SCID mice (table 2). For recipients of non-transduced control CD45RB\(^{\text{high}} \) CD4\(^{+} \) cells (Group 1), the course of the disease was typical: loss of weight within about 4 weeks after cell transfer and sickness from then on (figure 5). By contrast, the recipients of IL-10-GFP CD45RB\(^{\text{high}} \) CD4\(^{+} \) cells (Group 2) gained weight early on and were able to maintain their weight during the entire experiment (ANOVA repeated measure test \( p<0.001 \) Group 1 versus Group 2).

Transfer of CD4\(^{+} \) cells into SCID mice results in preferential repopulation of gut associated lymphoid tissues with immunocompetent CD4\(^{+} \) cells and subsequent expansion in the lamina propria of the host.\(^{26,27} \) FACS analysis of intestinal lymphocytes revealed that GFP fluorescent CD4\(^{+} \) cells were indeed present 12 weeks after transfer of IL-10-GFP CD45RB\(^{\text{high}} \) CD4\(^{+} \) cells (figure 6A). In addition, GFP fluorescent cells were found in the spleen, and the caudal and mesenteric lymph nodes (figure 6A). Using RT-
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Figure 6 IL-10-GFP expression in SCID mice reconstituted with IL-10-GFP CD45RB<sup>high</sup> CD4<sup>+</sup> cells

A) FACS analysis of colon, caudal and mesenteric lymph node and spleen cell suspensions was performed at sacrifice 9-12 weeks after transfer of control CD45RB<sup>high</sup> CD4<sup>+</sup> cells (Group 1; upper panel) or IL-10-GFP CD45RB<sup>high</sup> CD4<sup>+</sup> cells (Group 2; lower panel). The y-axis represents CD4 expression and the x-axis represents GFP fluorescence of gated viable lymphocytes. Representative images of 1 out of 14 mice (Group 1) and 12 mice (Group 2) are shown. Mean percentages of GFP<sup>+</sup> cells in the CD4<sup>+</sup> population are indicated.

B) The expression of the IL-10-GFP transgene was assessed by reverse transcription of total colonic RNA and PCR as described in material and methods. The upper panel shows a PCR of mice receiving IL-10-GFP CD4<sup>+</sup> cells (1) or non-transduced control CD4<sup>+</sup> cells (2) and PCR of a plasmid containing the construct IL-10-IRES-GFP (3). The reverse transcription reaction with mouse β-actin primers was performed parallel to a control of RNA quality (lower panel). The 879 bp and 628 bp bands are specific for the IL-10-GFP transgene and β-actin cDNAs respectively.
PCR, the IL-10-GFP encoding mRNA was detected in colons of mice that had been treated with IL-10-GFP CD4+ cells 15 weeks after cell transfer, but not in the control treated mice (figure 6B).

As expected, recipients of control cells (n=14) had increased colon weights, whereas colon weights of IL-10-GFP CD45RBhi CD4+ cell recipients (n=12) were virtually normal (346 ± 35 and 224 ± 18 mg respectively, p=0.006). Histological analysis indicated that the colons of control CD45RBhi CD4+ cell recipients had the typical features of inflammation in this model (histological score 2.4 ± 0.27, figure 7A). Conversely, only a minor influx of mononuclear cells and granulocytes in the colon was found after transfer of IL-10-GFP CD45RBhi CD4+ cells (histological score 1.0 ± 0.21, figure 7B). Indeed, the histological scores differed significantly between the two groups (p<0.001).

**IL-10-GFP CD45RBhi CD4+ cells influence pro-inflammatory cytokine production**

Cytokine levels (IL-2, IL-4, IL-5, IFN-γ and TNF-α) in spleen cell supernatants in the 2 groups did not diverge (data not shown). However, IFN-γ production by mesenteric lymph node cells was lower after transfer of IL-10-GFP CD45RBhi CD4+ cells (figure 8. Group 1: 2880 (59-12760) versus Group 2: 1080 (20-4171), p=0.05), indicating that these cells modulated cytokine production in the lymph nodes that drain the intestine.

TNF-α production by mesenteric lymph node cells did not differ (Group 1: 480 (20-2115) versus Group 2: 277 (20-672)). In addition, TNF-α concentrations in colon homogenates were lower in IL-10-GFP CD4+ cell recipients than in control CD45RBhi recipients (300 (260-441) versus 570 (472-669) pg/ml, p=0.05). IL-10 could be detected in 4 out of 5 colon homogenates of the IL-10-GFP CD45RBhi CD4+ cell treated mice but not in homogenates of control mice. In addition, IL-10 was detected in 2 out of 9 mesenteric lymph node and 6 out of 9 spleen cell supernatants of the mice transferred with IL-10-GFP CD45RBhi CD4+ cells but not in control mice.

Taken together, these results demonstrate that transduction of CD45RBhi CD4+ cells with the IL-10-GFP construct prevented induction of colitis in SCID mice. Moreover, the transduced cells migrated to the intestine and the draining lymph nodes, and influenced local production of pro-inflammatory cytokines.
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No effect of IL-10-GFP cells in TNBS colitis

Intrarectal administration of TNBS induces an acute Th1 cell mediated colitis with associated systemic features such as body weight loss. To assess the effect of IL-10-GFP transduced cells on the disease in this model, BALB/c mice (n=50) received a single intravenous injection with either non-transduced control splenocytes isolated from syngeneic donor mice or splenocytes transduced with IL-10-GFP (exp 1: 3.8x10^5 and exp 2: 0.5x10^5 GFP+ cells) on the day of the first TNBS administration. In these experiments, the cells were not sorted for CD4 or GFP expression. Nine days after transfer a small percentage (<1%) of the lymphocytes in the peripheral blood, spleen, and caudal lymph nodes expressed GFP. Transfer of IL-10-GFP transduced CD4+ cells had no effect on body weight, colon weight or histological score (data not shown).

Discussion

We here report that IL-10-GFP transduced CD4+ cells protect against transfer colitis in SCID mice, even when administration was delayed up to 14 days after transfer of CD45RB^hi CD4+ cells. We have shown that the protective effect of the IL-10-GFP CD4+ cells was a consequence of their regulating properties. This was demonstrated by the following findings. First, we included a control group to exclude the possibility that CD45RB^hi CD4+ cells present within the IL-10 transduced population prevented colitis. This control group was treated with non-transduced CD4+ cells that expressed CD45RB levels similar to transduced cells. Transfer of the control CD4+ cells was not protective and even slightly increased the severity of the colitis. The latter effect may have been caused by the presence of CD45RB^hi CD4+ cells in this population. The cytokine profile (IL-2, IL-4, IL-5, TNF-α, IFN-γ) of αCD3/CD28 activated and non-activated IL-10-GFP transduced and control CD4+ cells was identical, with the exception of IL-10. Secondly, human IL-10, which is bioactive in mice and which was incorporated in the retroviral vector to distinguish transgene expression from endogenous produced murine IL-10, was detected in the intestinal draining lymph nodes, spleen and colon of IL-10-GFP CD4+ cell treated mice. IL-10-GFP CD4+ cell transfer resulted in a decreased
Figure 7 Lack of intestinal inflammation in SCID recipients of IL-10-GFP CD45RB<sup>high</sup> CD4<sup>+</sup> T cells

Colon of SCID mice after transfer of control or IL-10-GFP CD45RB<sup>high</sup> CD4<sup>+</sup> cells. A) Severe colitis induced by control CD45RB<sup>high</sup> CD4<sup>+</sup> cells, characterized by a significant depletion of goblet cells and disorganization of the epithelial cells (mean score 2.4 ± 0.27). B) Some influx of mononuclear cells and granulocytes (mean score 1.0 ± 0.21)(p<0.001). Hematoxylin and eosin staining. Original magnifications: 33 x.

Figure 8. Decreased production of IFN-γ by mesenteric lymph node cells after transfer of IL-10-GFP CD45RB<sup>high</sup> CD4<sup>+</sup> cells

Mesenteric lymph node cells were isolated 9-12 weeks after transfer of control CD45RB<sup>high</sup> CD4<sup>+</sup> cells (Group 1) or IL-10-GFP CD45RB<sup>high</sup> CD4<sup>+</sup> cells (Group 2). Cells (1x10<sup>6</sup>/well) were stimulated with αCD3/CD28 and IFN-γ and TNF-α production were measured after 48 h in the supernatants. Mesenteric lymph node cells from IL-10-GFP CD45RB<sup>high</sup> CD4<sup>+</sup> reconstituted mice produced less IFN-γ compared with the control mice (p=0.05). Each symbol represents an individual mouse. Bars indicate median cytokine production for each group. Data are pooled from three separate experiments.
production of TNF-α in the colon and a decreased production of IFN-γ and/or TNF-α in the intestinal draining lymph nodes. Lastly, IL-10-GFP transduced CD45RB<sup>hi</sup> CD4<sup>-</sup> cells failed to induce colitis. A similar protective effect is shown in an earlier study, in which SCID mice did not develop colitis after reconstitution with CD45RB<sup>hi</sup> CD4<sup>-</sup> cells isolated from transgenic mice that expressed IL-10 under control of the IL-2 promoter. Together, these observations strongly suggest that the in vivo protective properties of the IL-10-GFP transduced CD4<sup>-</sup> cells resulted from increased expression of IL-10.

IL-10 is a regulatory cytokine that inhibits activation and effector function of T cells, monocytes and dendritic cells<sup>12</sup> and plays a central regulatory role in the immune responses of the intestine, limiting and ultimately terminating inflammatory responses. IL-10 has therefore been considered an attractive candidate for treatment of Crohn’s disease. Recent studies have indicated that systemic administration of rIL-10 in Crohn’s disease was relatively safe and well tolerated, but resulted in only a modest therapeutic benefit.<sup>13, 14</sup> In fact, higher doses of IL-10 were associated with systemic side effects such as fever, headache and malaise, most likely caused by an induction of IFN-γ production in vivo.<sup>15</sup> Moreover, systemic rIL-10 is rapidly cleared and mucosal bioavailability seems to be limited.<sup>16</sup> Our data indicate that transfer of IL-10 producing CD4<sup>-</sup> cells successfully inhibited inflammation in the local mucosal compartment, and prevented induction of colitis without interfering with systemic immune activation. This treatment was not associated with increased concentrations of circulating IL-10 and cytokine production by CD3/CD28 activated splenocytes was not altered. GFP fluorescent CD4<sup>-</sup> cells were present in the blood, spleen, colon, and lymph nodes draining the intestine, indicating that in effect the IL-10-GFP CD4<sup>-</sup> cells migrated to the mucosal compartment.

Although CD45RB<sup>hi</sup> and IL-10-GFP CD4<sup>-</sup> cells were injected at a ratio of 1:1 or 1:2, GFP<sup>+</sup> cells comprised only 6.5% of the circulating CD4<sup>-</sup> population two weeks following cell transfer. We hypothesize that the relative reduction of GFP<sup>+</sup> cells was a consequence of preferential expansion of the CD45RB<sup>hi</sup> CD4<sup>-</sup> cells. We have previously shown that human CD4<sup>-</sup> cells transduced with the same IL-10-GFP vector have a blunted proliferative response after stimulation.<sup>17, 18</sup> However, the percentage of circulating GFP<sup>-</sup> CD4<sup>-</sup> cells subsequently remained constant throughout the entire
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observation period. This finding argues against alternative explanations including silencing of the viral long terminal repeat (LTR) promotor\(^1\) and immunological clearance of GFP\(^+\) cells.\(^5\) \(^9\) Others have reported a sustained LTR driven transgene expression after retroviral transduction in mice for at least 16-20 weeks in immunocompetent mice.\(^41\) \(^42\)

We observed no therapeutic benefit from administration of IL-10 transduced cells in acute colitis induced by TNBS. It should be noted that systemic administration of rIL-10 (1-1000 \(\mu\)g/kg/day) did not affect the severity of mucosal necrosis in TNBS colitis in rats.\(^43\) Moreover, endogenous expression of colonic IL-10 is increased in TNBS colitis,\(^24\) \(^45\) and the immuno-regulatory cytokine response may already be maximally induced. In acute colitis, addition of exogenous rIL-10 may have no synergistic effects and may even be counteractive.\(^6\)

In other Th1-biased models of disease, such as experimental autoimmune encephalomyelitis and arthritis, antigen specific T cells have been used for the delivery of immuno-regulatory cytokine genes into inflamed areas.\(^7\) \(^14\) The triggering antigens in Crohn's disease or in animal models of inflammatory bowel disease have not been identified, and an antigen-specific T cell approach is thus not feasible. In the present study we have demonstrated that polyclonal, antigen non-specific, IL-10 transduced CD4\(^+\) cells had an anti-inflammatory effect. In addition, an earlier study reported that exposure to bacterial antigens is not required for the function of regulatory T cells in the transfer model, since CD45RB\(^{hi}\) cells isolated from germ-free mice were able to inhibit colitis.\(^41\) In fact, regulatory T cell clones can inhibit inflammatory responses via a mechanism called "bystander suppression". For example, regulatory T cells derived from CD4\(^+\) cells expressing a transgenic TCR specific for ovalbumin inhibited the function of T cells responding to unknown intestinal antigens.\(^3\) Hence, the effector function of IL-10 transduced CD4\(^+\) cells can be antigen non-specific.

Different and mutually not exclusive mechanisms of the effector function of regulatory T cells have been postulated.\(^11\) Interaction of regulatory T cells with dendritic cells following priming may impair the costimulatory capacity of dendritic cells via inhibition of costimulatory molecule expression. Alternatively, regulatory T cells may interfere with proliferative and homing capacity of activated T cells, preventing the
accumulation of pathogenic T cells in the intestine. Finally, anti-inflammatory cytokines produced by regulatory T cells in the intestine may prevent the release of chemokines and pro-inflammatory cytokines by macrophages, inhibiting the progression of the inflammatory response. In line with these hypotheses, we have demonstrated that human IL-10-GFP transduced CD4+ cells inhibit proliferation of autologous responder CD4+ cells and IL-12 production by DCs.

Several other delivery systems have been used to locally deliver IL-10 to the intestine, such as adenoviral vectors or cationic lipids complexed to immunoregulatory genes, but a common major disadvantage of these methods is that transgene IL-10 expression is transient. Alternatively, bacteria can be genetically engineered to express IL-10. Proof of concept was obtained in a recent study showing that daily administration of IL-10 expressing Lactococcus lacti prevented colitis in two models of experimental colitis. This method required a much lower amount of IL-10 compared with intraperitoneal rIL-10 administration. However, the IL-10 producing Lactococci only transiently increase mucosal IL-10 concentrations, as these engineered bacteria do not colonize the intestine. Moreover, at present it is unclear whether these approaches will induce the development of T cells with a regulatory function. *Ex vivo* retroviral gene transfer combines non-direct exposure of the organism to the genetic engineered viral particles with efficient and sustained gene expression.

In conclusion, we have demonstrated that experimental colitis is controlled by CD4+ cells transduced with IL-10, without systemic immune suppression. We propose that the genetic engineering of peripheral CD4+ cells using retroviral IL-10 gene transfer may become a clinically viable approach to the treatment of Crohn's disease and other T cell mediated inflammatory diseases. Our data suggest that this approach would be best suited to maintenance treatment rather than to induction of remissions in severely active Crohn's disease.

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