Interleukin 10 gene therapy for Crohn's disease
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Chapter 5

Generation of regulatory gut-homing human T lymphocytes using ex vivo interleukin 10 gene transfer

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resubmitted with revisions to Gastroenterology
Chapter 5

Abstract

Background & Aims: Systemic treatment of Crohn's disease patients using rIL-10 has not resulted in relevant therapeutic benefit presumably due to limited bioavailability and unexpected pro-inflammatory effects of high-dose rIL-10. In vivo gene transfer of the IL-10 gene to gut-homing CD4+ cells may lead to improved long-term management.

Methods: PBMCs were transduced with a retroviral vector containing the IL-10 and GFP gene or a control vector containing GFP only. Transduced CD4+ cells were sorted and maintained in culture for phenotypic and functional analysis.

Results: Stimulated IL-10-GFP CD4+ cells produced significant higher levels of IL-10 than control cells for at least 4 months. The IL-10 transgene was biologically active and decreased proliferation of IL-10-GFP CD4+ cells as well as expression of MHC class II, proliferation of autologous responder cells and IL-12 production by dendritic cells. The majority of transduced CD4+ cells had a gut-homing potential, since they expressed the mucosal integrin α4β7, and displayed efficient binding to MAdCAM-1-expressing cells in vitro.

Conclusions: Transduction of peripheral blood CD4+ lymphocytes with IL-10 results in a regulatory phenotype. The use of regulatory gut-homing human CD4+ cells may provide a novel approach to local delivery of immunomodulatory signals to the intestine in Crohn's disease.
Introduction

Crohn's disease is characterized by a chronic inflammation of the gastro-intestinal tract. In most patients with active disease remissions can be induced using corticosteroids or tumour necrosis factor α (TNF-α) neutralizing antibodies. However, such remissions are oftentimes short-lived and about 50% of the patients treated with corticosteroids show an early relapse. Repeated infusions of TNF-neutralizing antibodies can maintain the therapeutic effects in a significant number of patients, but long-term administration is costly and can be immunogenic. Hence, there is a continued need for effective maintenance therapy in Crohn's disease.

Substantial evidence indicates that activated CD4+ T helper 1 (Th1) cells, which produce pro-inflammatory cytokines such as TNF-α, mediate mucosal inflammation and tissue damage. A recently recognized subset of CD4+ cells deserves special attention as it predominantly produces IL-10. IL-10 inhibits a broad array of immune parameters, including activation and effector function of T cells, monocytes and dendritic cells (DCs), limiting and ultimately terminating inflammatory responses. These IL-10 producing cells are named regulatory T cells, because of their immunosuppressive effects both in vitro and in experimental colitis in vivo. Regulatory T cells produce little IL-2, no IL-4 and the production rate of IL-5, IFN-γ and transforming growth factor beta (TGF-β) is similar to naïve T cell clones. The mechanisms by which regulatory T cells mediate immunosuppressive activities in vivo are still unknown, but some regulatory T cell populations are dependent on IL-10 for their function.

It has been reported that subcutaneous administration of recombinant (r)IL-10 in Crohn's disease is safe, well tolerated and has some therapeutic efficacy. However, high concentrations of rIL-10 (e.g. subcutaneous 20 μg/kg) are associated with adverse effects such as headache and fever as well as overproduction of pro-inflammatory cytokines. Moreover, studies in healthy human volunteers and Crohn's disease patients demonstrated that systemically administered rIL-10 has a restricted half live (2.5 hours) and may have limited mucosal bioavailability. The limitations of systemic rIL-10 therapy might be overcome by targeting IL-10 production to the intestine using IL-10 gene transfer. CD4+ cells are ideal carriers for the IL-10 gene, because subsets of
CD4+ cells have the capability to “home” to the intestine, which is mainly mediated by the interaction of the integrin α4β7 and the mucosal addressin MAdCAM-1.21

Because of the central role of IL-10 in controlling intestinal inflammation, we have sought to engineer human CD4+ cells that constitutively produce IL-10 using a retroviral vector containing the IL-10 cDNA. We investigated whether these CD4+ cells produced more IL-10 compared with control cells and determined whether IL-10 production was activation-dependent. Furthermore, we studied the biological activity of the IL-10 transgene and the gut-homing potential of the transduced CD4+ cells.

Material and methods

Vectors and viral production

The LZRSpBMN-IRES-GFP retroviral plasmid (referred to as GFP) has been previously reported.22 Briefly, the GFP complementary DNA (cDNA) (Clontech, Palo Alto, CA, USA) was cloned downstream of an internal ribosome entry site (IRES) in the LZRS retroviral vector (kindly provided by G. Nolan)23 and expressed from the retroviral long terminal repeat (LTR) from the Moloney Murine Leukemia Virus (MMLV). A 536-bp PCR fragment containing the human IL-10 cDNA24 was amplified from human cDNA with primers containing a BamHI (fw: GGATCCACCATGACGCTGCTCTGT) and a Xho I (rv: CTCGAGTCAGTTTCGTTACCTCTATTGTTCATGT) restriction site. 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-IL-10-IRES-GFP and referred to as IL-10-GFP.

293T Phoenix packaging cells expressing the amphotropic envelope (kindly provided by G. Nolan) were cultured in Dulbecco’s modified Eagle’s medium (GibcoBRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS) (Biowhittaker, Walkersville, MD, USA), 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (GibcoBRL) under standard conditions (37°C, 5% CO₂). The packaging cells were transfected with the retroviral plasmids using calcium phosphate precipitation.
Engineered T cells for IL-10 delivery to the gut

Viral supernatants were harvested after two weeks of puromycin selection (Clontech), filtered using a 0.45 µm filter (Nalge, Rochester, NY, USA) and stored at -80°C. Virus titres were determined using NIH 3T3 lines, as previously described. Virus stocks with titres of $10^6$-10$^7$ IU/ml were used for transduction of human T cells.

Retroviral transduction of human T cells

PBMCs were obtained from healthy adults after informed consent by Ficoll HyPaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. PBMCs (1x10$^6$/well) were seeded in 24 well plates (Costar Europe Ltd, Badhoevedorp, The Netherlands) and cultured under standard conditions in Iscove's modified Dulbecco's medium (IMDM, Biowhittaker) supplemented with 10% human serum (Biowhittaker), 2 mM glutamine, and 1% antibiotic-antimycotic solution (GibcoBRL). Cells were stimulated with 1 µg/ml of phytohemagglutinin (PHA, Murex Diagnostics, Dartford, UK) and 10 U/ml of recombinant human IL-2 (Chiron, Amsterdam, The Netherlands) under standard conditions. After 48 hours (h), 5x10$^5$ cells were seeded in 6 well RetroNectin-coated plates (Takara, Otsu, Japan), and transduced overnight with 1 ml of retroviral supernatant, then, cells were washed and transferred to 24 well plates in supplemented IMDM. After at least 2 days the transduced cells were analyzed by flow cytometry for GFP expression. T cells (3x10$^5$/well) were expanded in the presence of a feeder mixture consisting of irradiated allogeneic human PBMCs of two donors (1x10$^6$/ml each), 1x10$^5$/ml of irradiated JY cells, 100 ng/ml PHA and 10 U/ml IL-2.

Purification of transduced CD4$^+$ T cells

To obtain a purified CD4$^+$ cell subset, eight to ten days after transduction, cells were stained with CD4-PE (Coulter-Immunotech, Marseille, France) and sorted in a FACS Vantage Cell Sorter (Becton Dickinson, C.A. USA) into subsets of CD4 and GFP expressing cells. After sorting, a double-positive population was purified to at least 90%. The sorted GFP$^+$ CD4$^+$ cells were expanded and cultured in the feeder mixture for further analysis.
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Cytokine measurement

The cytokine profile of transduced CD4⁺ cells was determined 10 days after the addition of a feeder mix. Cells were washed in medium and 1x10⁵ cells/well were plated out on 96-well round bottom plates (Costar) in a final volume of 200 μl of medium in the absence or presence of immobilized anti-(α)CD3 (clone SPV-T3b) and 2 μg/ml soluble αCD28 monoclonal antibodies (mAb) (clone CLB-CD28/1 ascites fluid, CLB, Amsterdam, The Netherlands). After a 24 and 48 h incubation, cell-free supernatants collected from 4 wells were pooled and stored at -20°C until further use. Cytokine levels in culture supernatants were measured in duplicate by a sandwich enzyme-linked immunosorbent assays (ELISA) for IL-2 (R&D systems, Abingdon, UK), IL-4, IL-10, IFN-γ and TNF-α (CLB) and IL-5 (Pharmingen, San Diego, CA, USA), according to manufacturer's instructions.

An IL-10 secretion assay (Miltenyi Biotec, Auburn, CA, USA) was performed according to the instructions of the manufacturer, after 1-month culture. In short, transduced CD4⁺ cells were stimulated for 24 hour with αCD3/CD28 as described above. Subsequently, cells were harvested, washed and an IL-10 specific catch reagent (Miltenyi Biotec) was added and incubated for 5 min on ice. After a 45 min secretion period at 37°C, cells were washed and stained with a PE labelled IL-10 detection antibody (Miltenyi Biotec) and Cy5-CD4 (Beckman-Coulter, Fullerton, CA, USA) for FACScan (Becton Dickinson) analysis.

Cell proliferation assays

The proliferation rate of the transduced CD4⁺ T cells was measured at the same time point (48 h) and under equal conditions (+/- activation) as cytokine production. During the last 6 h of culture, cells were incubated with 0.25 μCi/well ³H-thymidine (Amersham, Les Ulis, France). At 48 h the cells were lysed, the homogenates were harvested on a filter and the incorporated radioactivity was measured on a Topcount scintillation counter (Packard Instruments, Meriden, CT, USA). The proliferation rate is given as stimulation index, calculated as incorporated radioactivity of αCD3/CD28 activated CD4⁺ cells divided by the incorporated radioactivity of resting CD4⁺ cells.
Engineered T cells for IL-10 delivery to the gut

In separate experiments, the proliferation rate of increasing numbers (up to 5x10^4/well) of sorted transduced CD4^+ cells was measured after stimulation with allogeneic monocytes (1x10^4/well). Monocytes were isolated using Percoll (Pharmacia, Uppsala, Sweden) density centrifugation (90% CD14^+). Cells were cocultured in a final volume of 200 µl of medium in 96-well round bottom plates (Costar).

In coculture experiments, autologous responder cells (cultured CD4^+ cells and fresh PBMCs) (5x10^4/well) were isolated as described above and stimulated with monocytes (1x10^4/well). Cell proliferation assays were conducted in the presence of increasing concentrations of rIL-10 (0 to 20 ng/ml, Strathmann Biotec, Hannover, Germany) or supernatants of transduced cells (1x10^3/well), collected after 48 h of αCD3/CD28 stimulation. Transduced CD4^+ cells were tested for their ability to suppress the proliferation of autologous CD4^+ cells and PBMCs. For suppression, increasing numbers (up to 5x10^4/well) of irradiated IL-10-GFP or control transduced CD4^+ cells were added. Cells were cocultured in a final volume of 200 µl of medium in 96-well round bottom plates (Costar). After 3 days, wells were pulsed for 16 h with 0.25 µCi/well ^3H-thymidine (Amersham) and the incorporated radioactivity was determined as described.

Analysis of IL-12 production by dendritic cells

DCs were generated from PBMCs as described previously. In brief, monocytes isolated by Percoll density gradient centrifugation were cultured (0.5x10^6/well) in IMDM supplemented with 10% FCS (Hyclone, Logan, UT) and GM-CSF (500 U/ml, Schering-Plough, Uden, The Netherlands) and IL-4 (250 U/ml, PBH, Hannover, Germany). After 6 days of culture, irradiated IL-10-GFP or control transduced CD4^+ cells (0.5x10^6 cells/well) were added in duplicate wells in the presence of GM-CSF (1000 U/ml). On day 8 DCs were harvested, washed extensively and plated (2x10^4/well) in duplicate with CD40L expressing cells (2x10^4/well, CD40L-transfected J558L cell line kindly provided by Peter Lane, University of Birmingham, Birmingham, UK) in 96-well plates (Costar) in IMDM containing 10% FCS (Hyclone). DCs were cultured in the presence or absence of IFN-γ (1000 U/ml) in a final volume of 200 µl for 24 h. IL-12p70 levels were analyzed in culture supernatants in duplicate by ELISA (R&D systems, Abingdon, UK), according to manufacturer’s instructions.
Chapter 5

Cell surface phenotyping

The expression of several activation and differentiation markers was studied on resting CD4+ cells after transduction. Cells were harvested, washed with cold FACS buffer (0.5% BSA, 0.01% NaN₃, and 0.3 mM EDTA in PBS, pH 7.4) and incubated for 20 min on ice with the following mAbs: Cy5-CD4 (Beckman-Coulter), PE-CD154 (Immunotech, Hamburg, Germany), or unlabelled CD18, CD25, CD27, CD28 (all CLB), CD44 (Pharmingen), CD45RO (Dako, Glostrup, Denmark), CD62L (Becton Dickinson), CD152 (Immunotech), α4β7 (gift from Leukosite, Cambridge, MA, USA), CXCR3 (Pharmingen), Q5/13 (MHC cl II, gift from S. Feronne, Medical College, Valhalla, NY, USA) followed by PE- rabbit-anti-mouse F(ab)_2 (Dako). Isotype controls included PE- and Cy5-conjugated IgG (Immunotech). Stained cells were analyzed using a FACSscan (Becton Dickinson) and the data were processed with CellQuest computer software.

Adhesion assay

We investigated whether transduced CD4+ cells were able to bind to the mucosal addressin MAdCAM-1 measuring their adhesion to 293T cells stably expressing the murine MAdCAM cDNA (kindly provided by Dr. S. Fong, Genentech Inc., San Francisco, CA, USA). Transduced CD4+ cells were labelled with 50 μM carboxyfluorescein diacetate succinimidyl ester (CFDSE: Molecular probes, Eugene, Oregon, USA) at 37°C for 10 min, washed in ice-cold PBS and resuspended in adhesion assay medium consisting of Dulbecco’s without phenol red (GibcoBRL) supplemented with 10% FCS (Biowhittaker), 2 mM glutamine, and 1% antibiotic-antimycotic solution (GibcoBRL). The CFDSE-labelled CD4+ cells were preincubated with the following mAbs for 20 min before the adhesion assay α4β7 (Act-1, gift from Leukosite), MAdCAM-1 (MECA-367, gift from Dr. R. Mebius, Vrije Universiteit Medical Center, Amsterdam, The Netherlands) and a control antibody (145 2C11, gift from Dr. R. Mebius). MAdCAM-1 overexpressing and control 293T cells (5x10⁴/well) were plated on 96-well plates (Costar) and cultured for 24 h. Labelled CD4+ cells (1x10⁵/well) were added in quintuplicate and incubated for 30 min at 37°C. Non-adherent CD4+ cells were washed with adhesion assay medium. Fluorescence was measured in a Cytofluor plate reader (Perceptive Biosystems, Framingham, MA, USA) before and after washing. The
adherence was calculated as the percentage of fluorescent cells remaining after washing. The maximum binding was calculated as the difference in adherence to MAdCAM-1 transfected and control 293T cells.

**Statistical analysis**

Differences in cytokine production and proliferation between different cell populations were analyzed by the non-parametric Mann-Whitney U test. Differences in proliferation were also tested by analysis of variance (ANOVA) for repeated measures. Results are expressed as the mean ± SEM. A two-tailed p value of less than 0.05 was considered to represent a significant difference.

**Results**

**Efficient transduction and stable expression of GFP by transduced CD4+ T cells**

Fresh PBMCs were stimulated with PHA and IL-2 for 48 h. Stimulated PBMC populations routinely consisted of >90\% CD3+ cells (data not shown). The PBMCs were subsequently transduced with IL-10-GFP or GFP retroviral constructs and the transduction efficiencies were determined after 48 h on the basis of GFP expression in viable cells. Transduction efficiencies ranged from 13 to 48\%, with a mean of 26 ± 3\% (16 transductions performed in 8 different donors). PBMCs were sorted on the basis of CD4 and GFP expression using a FACS Vantage flow cytometer (figure 1A). GFP expression correlated well with IL-10 secretion as shown in figure 1B. Upon reanalysis, the sorted GFP+ CD4+ cells had a purity of 93 ± 2\%. This percentage remained constant during the entire period of cell culture (more than 4 months), indicating that the transgene was stably integrated and expressed. This is in agreement with a previous study describing stable transgene expression in human T cells for up to 6 months using the same LZRS vector containing telomerase.2
Figure 1  FACS analysis and sorting of IL-10-GFP transduced CD4+ cells

A) FACS analysis and sorting of CD4 and GFP expressing cells was performed 48 h after transduction with the IL-10-GFP containing MMLV vector. The y-axis represents CD4 expression and the x-axis represents GFP fluorescence. Percentages of GFP+ CD4+ cells are indicated before (left panel) and after sorting (right panel). This is a representative image of 8 independent transductions.

B) An IL-10 secretion assay was performed with the sorted control GFP (left panel) and IL-10-GFP CD4+ cells (right panel) one month after the transduction as described in the materials and methods section. IL-10 secreting cells were stained with a PE labelled IL-10 detection antibody. The y-axis represents the IL-10 secretion and the x-axis represents the GFP fluorescence. Percentages of GFP+ IL-10+ cells are indicated for the control GFP (left panel) and IL-10-GFP CD4+ cells (right panel).
Engineered T cells for IL-10 delivery to the gut

Figure 2 IL-10 production by transduced CD4+ cells

CD4+ cells (1x10^5/well) isolated from a healthy donor were cultured in the absence (A) or presence (B) of αCD3/CD28 mAbs for a period of 48 h on a 96-well plate as described in the materials and methods section. The supernatants were harvested and IL-10 concentrations were measured by ELISA at different time-points (2, 6, 10 and 17 weeks) after retroviral transduction. The production of IL-10 is expressed in nanograms of IL-10 per ml per 48 h (ng/ml/48 h). Each value is the mean of duplicate measurements ± SEM.

Cytokine production analysis

We first quantified the amount of IL-10 in the supernatants of sorted GFP+ CD4+ cell cultures from one healthy donor. The production of IL-10 by IL-10-GFP CD4+ cells was measured 2, 6, 10 and 17 weeks after transduction. It is clear from Figure 2A that αCD3/CD28 activated IL-10-GFP CD4+ cells had a high and stable IL-10 production compared with control GFP CD4+ cells.

As previously reported, transgene expression increases substantially when retroviral transduced lymphocytes are reactivated by CD3/CD28 engagement.20 IL-10-GFP CD4+ cells upregulated IL-10 expression indeed after αCD3/CD28 stimulation significantly compared with resting cells. A representative example is given in figure 2A and B. These results were highly reproducible when studied in 8 other donors: stimulated IL-10 production was 12.9 ± 2.1 (figure 3) versus 0.59 ± 0.06 ng/ml/48 h by resting
Figure 3 Cytokine profile of transduced CD4+ cells

Cytokine concentrations were measured by ELISA in the supernatants of sorted GFP-CD4+ cells after transduction with either GFP or IL-10-GFP, as described in the legend of figure 2. IL-2 and IL-4 levels are given after 24h of αCD3/CD28 stimulation, as these cytokines are rapidly consumed. The other cytokines are given after 48h of stimulation, because their production peaked at this time point. Results of pooled data from 8 donors are expressed as mean ± SEM and * represents a significant difference (p=0.002).

IL-10-GFP CD4+ cells (p=0.003). ACD3/CD28 stimulation of control GFP CD4+ cells also led to a higher IL-10 expression (figure 3) compared with non-stimulated CD4+ cells (2.08 ± 0.61 versus 0.19 ± 0.1 ng/ml/48 h, p=0.002), though these levels were evidently lower than the IL-10 levels in the supernatants of the IL-10-GFP CD4+ cells. To examine whether the cytokine profile was affected by transduction with IL-10-GFP, we measured the production of Th1 (IL-2, IFN-γ and TNF-α) and Th2 cytokines (IL-4, IL-5 and IL-10) after αCD3/CD28 stimulation (figure 3). IL-2 and IL-4 levels are provided after 24h of stimulation, as they are rapidly consumed. The levels of the other cytokines are shown after 48h stimulation, because their production peaked at this time point. IL-10-GFP CD4+ cells produced approximately 6-fold higher levels of IL-10 than control GFP CD4+ cells (p=0.002), but there was no significant difference in the production of Th1 and other Th2 cytokines (figure 3). Remarkably, activation of both cell types resulted in high levels of IFN-γ.
Engineered T cells for IL-10 delivery to the gut

**Figure 4** Autocrine inhibition of IL-10-GFP transduced CD4⁺ cell proliferation

A) The proliferation of transduced CD4⁺ cells from 2 donors was measured after a 48 h incubation in the presence or absence of αCD3/CD28 mAbs. Incorporation of ³H-thymidine was determined after a pulse ³H-thymidine during the last 6 h of culture. The proliferation rate is given as stimulation index, calculated as incorporated radioactivity of αCD3/CD28 activated CD4⁺ cells divided by the incorporated radioactivity of resting CD4⁺ cells. This experiment, performed in parallel with the cell cultures used for cytokine detection, represents one of 3 independent experiments yielding similar results. Each value is the mean of measurements ± SEM and * indicates a significant difference (p=0.004).

B) Increasing numbers of irradiated or non-irradiated IL-10-GFP and control CD4⁺ cells (0 to 5x10⁴ cells/well) were stimulated for three days with allogeneic monocytes (1x10⁵/well). Incorporation of ³H-thymidine was determined after a pulse ³H-thymidine during the last 18 hours of culture. Each value is the mean of triplicate measurements ± SEM and analysis of variance (ANOVA) for repeated measures indicated that proliferation of the two cell types differed significantly from 6.3x10³ cells/well and onwards (p<0.001). The results are representative of 3 independent experiments.

**IL-10-GFP CD4⁺ cells have a blunted proliferative response and suppress proliferation of autologous cells in a paracrine way**

An important feature of IL-10 is the inhibition of T cell proliferation. Yet, to test this biological activity, we performed a ³H-thymidine incorporation assay on our transduced
cell cultures in parallel to the cytokine production assays after 48 h of αCD3/CD28 activation. Figure 4A shows the stimulation index of 2 representative donors of a total of 6. The IL-10-GFP CD4+ cells had a reduced proliferative response to αCD3/CD28 activation in comparison to control GFP CD4+ cells. Similar results were obtained after 72 h of αCD3/CD28 activation (data not shown). These data indicate that the increased production of IL-10 by activated IL-10-GFP CD4+ cells, as shown in figure 3, reflects an increased IL-10 production per cell, as it could not be ascribed to an increased number of cells due to proliferation. In addition, IL-10-GFP CD4+ cells had a blunted proliferative response when stimulated with allogeneic monocytes in sharp contrast to the control GFP CD4+ cells (figure 4B).

To obtain the effect of rIL-10 on alloantigen induced proliferation of responder cells (e.g., cultured CD4+ cells and fresh PBMCs), these cells were stimulated with purified allogeneic monocytes. In figure 5A, it is shown that rIL-10 inhibited the proliferative responses in a dose-dependent fashion. Significant inhibitory effects were already observed at rIL-10 concentrations of 1.25 ng/ml (p=0.049 versus no rIL-10 added) and 2.5 ng/ml (p=0.050 versus no rIL-10 added) in the CD4+ cells and PBMCs, respectively. We compared this effect of rIL-10 with supernatants collected from IL-10-GFP or control GFP CD4+ cells after 48 h of αCD3/CD28 stimulation, containing IL-10 concentrations of 4.1 ± 0.2 and 0.4 ± 0.07 ng/ml, respectively. The supernatants from the IL-10-GFP CD4+ cells suppressed the proliferation of the autologous CD4+ cells and PBMC responder cells by an average of 61% and 18%, respectively (figure 5B).

We next tested the ability of the transduced cells to suppress the proliferative responses of autologous responder cells to alloantigens in coculture experiments. Responder cells were stimulated with monocytes and different numbers of irradiated transduced cells. As shown in figure 5C at a ratio of 1:2 (IL-10-GFP CD4+ cells: responder cells), the proliferation of CD4+ cells and PBMCs was inhibited by an average of 67% and 38%, respectively. At a ratio of 1:1 the proliferative responses were even more reduced (data not shown) compared with the control GFP CD4+ cells. From this we conclude that the expression of the IL-10 transgene leads to secretion of IL-10 that is biologically active in both an autocrine and paracrine fashion.
Engineered T cells for IL-10 delivery to the gut

Figure 5 Inhibition of the proliferative response of autologous responder cells to alloantigens in the presence of rIL-10, the supernatants or transduced cells

Cultured CD4+ cells or freshly isolated PBMCs (5x10^5/well) were stimulated for three days with allogeneic monocytes (1x10^5/well). A) In the presence of increasing concentrations of rIL-10 (0 to 20 ng/ml). B) In the presence of supernatants of the transduced CD4+ cells (1x10^5/well), harvested after 48 h αCD3/CD28 stimulation. C) In the presence of irradiated IL-10-GFP or control CD4+ cells (2.5x10^5/well). Incorporation of 3H-thymidine was determined after a pulse 3H-thymidine during the last 18 h of culture. Each value is the mean of triplicate measurements ± SEM and * represents a significant difference (p<0.05). The results are representative of 3 independent experiments.

IL-10-GFP CD4+ cells reduce IL-12 production by dendritic cells

We next investigated whether interaction of the IL-10-GFP transduced CD4 cells with DCs influenced the capacity of DCs to produce IL-12, since it is known that rIL-10 suppresses IL-12 production by DCs.52 To this end, immature DCs were cocultured for 48 h with irradiated IL-10-GFP or control GFP CD4+ cells followed by thorough washing. DCs were incubated for another 24 h in the presence or absence of IFN-γ. In three independent experiments coculture of IL-10-GFP CD4+ cells with DCs resulted in a 8-fold decrease in IL-12p70 production (mean 79 ± 21 pg/ml/24 h) compared with coculture with control GFP CD4+ cells (mean 436 ± 75 pg/ml/24 h, p=0.004). DCs activated with IFN-γ also produced less IL-12p70 after coculture with IL-10-GFP CD4+ cells (162 ± 46 versus 451 ± 74, p= 0.01). These combined results indicate that the suppressive activities of IL-10-GFP CD4+ cells extend from CD4+ cells and monocytes to DCs.
Table 1 Phenotypic analysis of transduced CD4⁺ cells

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Sorted GFP⁺CD4⁺ cells were stained with mAbs directly or indirectly labelled with PE or Cy as described in material and methods and analyzed on a FACScan. Gates were set to contain live cells only. Indicated are the levels of expression (mean fluorescence intensity: MFI) in four categories as follows: MFI<10, ±; MFI 10-200, +; MFI 200-500, ++; MFI >500, ++++. The mean MFI of 2 donors (the same as used in figure 4) analyzed in one experiment is shown and the results are representative for 8 donors.

Phenotype of IL-10 transduced T cells

We studied the cell surface phenotype of resting transduced cells by FACScan analysis. As expected, MHC class II antigen was downregulated on IL-10 transduced CD4⁺ cells (table 1). On the other hand, IL-10-GFP transduction did not influence the expression of other activation markers (table 1). CD154 (surface CD1A-4), important for downregulation of activated CD4⁺ cells, was not detectable. Essentially all transduced CD4⁺ cells expressed the CD45RO marker of memory T cells.

Since the IL-10 transduced CD4⁺ cells should be specifically directed to the intestine after reinfusion to a patient, we studied the expression of different adhesion markers. The CD18, CD44, CD62L, and α4β7 adhesion molecules direct lymphocytes respectively to inflammatory sites, high endothelial venules (HEVs), peripheral lymph nodes, and to the intestine. We observed an intermediate expression of CD62L (L-selectin) and high expression of CD18 (HEV-1) and CD44 on all transduced cells.
Engineered T cells for IL-10 delivery to the gut

CD4+ T cells:

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<td>control GFP</td>
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**Figure 6** High expression of α4β7 on fresh and cultured human CD4+ cells

Flow cytometry analysis of α4β7 expression on A) fresh CD4+ cells of a healthy donor, cultured CD4+ cells that were either B) non-transduced or C) transduced with the control GFP vector or D) the IL-10-GFP vector. The y-axis represents the relative cell number (counts) and the x-axis represents α4β7 expression. The results shown are representative for 8 donors.

Importantly, expression of the principal gut-homing molecule α4β7 was high on both transduced and non-transduced cultured CD4+ cells as compared with freshly isolated CD4+ cells (figure 6). The expression of the Th1 related molecule CXCR3 was comparable in both culture conditions.

**α4β7 mediates adhesion of transduced CD4+ T cells to MAdCAM-1**

Since adhesion of gut-homing T cells to the intestinal HEVs depends on the expression of a functional form of α4β7 on the cell surface, we investigated whether adhesion of transduced CD4+ cells to MAdCAM-1 transfected 293T cells was α4β7-dependent. The GFP and IL-10-GFP CD4+ cells adhered more efficient to the MAdCAM-1 transfected
Figure 7 Inhibition of CD4⁺ cell adhesion to MAdCAM-1 transfected 293T cells by α4β7 and αMAdCAM-1 mAbs

Adhesion of sorted CD4⁺ cells, transduced with GFP (1 donor) or IL-10-GFP (2 donors), to MAdCAM-1 transfected 293T cells was determined after preincubation with or without the following mAbs: 145 2C11 (control), act-1 (α4β7) or MECA-367 (αMAdCAM-1). Fluorescent-labelled CD4⁺ cells were added to a 96-well plate that contained confluent MAdCAM-1 transfected 293T cells or control 293T cells. Relative binding inhibition was referred to the maximum binding achieved in the absence of mAbs (as described in materials and methods).

As shown in figure 7, adhesion of IL-10-GFP and control GFP CD4⁺ T cells to MAdCAM-1 transfected cells was partially blocked by pre-treatment with mAb against α4β7 or MAdCAM-1, but not by pre-treatment with a control antibody. These results indicate that the expressed α4β7 mediated binding to MAdCAM-1, and T cells retain the capacity to bind to MAdCAM-1 after retroviral transduction.
Discussion

In this study, we aimed to genetically modify human CD4+ cells in vitro in order to induce increased production of IL-10. We hypothesized that such T cells would acquire a regulatory phenotype, ultimately aiming at using these cells therapeutically in Crohn's disease and possibly other Th1 cell mediated inflammatory diseases.

PBMCs obtained from healthy donors were transduced using a MMLV-based retroviral vector containing IL-10-GFP or GFP alone as a control. In accordance with other studies,\textsuperscript{29-42} we demonstrate an efficient retroviral gene transfer to primary CD4+ cells. CD3/CD28 stimulated CD4+ cells transduced with IL-10-GFP produced 6-fold more IL-10 as compared with CD4+ cells transduced with GFP only and IL-10 and GFP transgene expression in vitro was stable for a period of more than 4 months. This finding is in agreement with recent publications on retroviral transduction of T cells that report long-term transgene expression in vitro.\textsuperscript{28, 41}

The efficiency and longevity of IL-10 transgene expression in vivo remains to be determined as it depends on both the survival of the transduced T cells and the LTR viral promoter activity. MMLV-based viral vectors have shown a propensity for the acquisition of de novo methylation in vitro, causing silencing of transgene expression.\textsuperscript{44, 45} However, long term survival of T cells after adoptive transfer and transgene expression has been repeatedly reported. For example, the survival of T cells transduced with adenosine deaminase gene, the herpes simplex type I thymidine kinase gene or the γc cytokine receptor subunit gene varied between several months\textsuperscript{46-47} and 4 years.\textsuperscript{48} In the latter study, two patients with SCID-X1, a disease with a cytokine receptor γ chain (γc) gene deficiency, were treated with retrovirally transduced hematopoietic progenitor cells overexpressing the missing gene. After a 10-month follow-up, the disease phenotype was corrected with normal counts of functional, γc transgene-expressing T and NK cells.\textsuperscript{48} In addition, in a model of experimental colitis we demonstrated that murine T cells, which were transduced with the same IL-10 vector as used in this study, survived more than 15 weeks, effectively producing IL-10 throughout the study period.\textsuperscript{59} Hence, we expect IL-10 transduced T cells to have a long half-life in vivo.
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Downregulation of MHC class II expression and inhibition of T cell proliferation are two major immunosuppressive activities of IL-10. As expected, MHC class II expression was decreased on the surface of IL-10-GFP CD4⁺ cells. Analysis of the proliferative responses of the transduced cells revealed that IL-10-GFP transduction of CD4⁺ cells resulted in a reduction of their proliferative capacity following polyclonal activation or stimulation with alloantigens. In addition, IL-10-GFP CD4⁺ cells suppressed the proliferation of autologous responder cells, indicating that these cells have immunomodulatory properties.

Another biologically relevant finding was that IL-10-GFP CD4⁺ cells potently downregulated IL-12 production by DCs. IL-12 is a major Th1 inducing cytokine and in Crohn's disease both IL-12 and its receptor are upregulated within the inflamed mucosa. The functional importance of mucosal IL-12 production in Crohn's disease is strongly supported by the finding that IL-12 contributes to the preferential expansion of IFN-γ secreting cells. Studies in murine models have demonstrated that neutralization of IL-12 leads to complete recovery of experimental colitis. Hence, inhibition of Th1 cell development via IL-12 downregulation and control of CD4⁺ cell proliferation by IL-10-GFP CD4⁺ cells are expected to control mucosal immune responses in Crohn's disease.

In the absence of TCR stimulation, IL-10 production by control GFP CD4⁺ cells was undetectable, whereas resting IL-10-GFP CD4⁺ cells made some IL-10. Stimulation with αCD3/CD28 significantly induced IL-10 production by these cells. Our finding adds to an increasing number of publications reporting that activation of T cells influences transgene expression of MMLV-based vectors. It is known that the MMLV 5'LTR promoter driving transgene expression can be influenced by the activation state of the T cell. This finding confers promising perspectives to the use of MMLV transduced CD4⁺ cells in patients with Crohn's disease, because focal inflammation in these patients will potentially stimulate the local expression of the transgene.

We subsequently investigated whether IL-10 gene transfer influenced the cytokine profile of transduced T cells. The production of IL-2, IL-4, IL-5, TNF-α and IFN-γ by αCD3/CD28 activated and non-activated transduced cells was identical, with the exception of IL-10. This is consistent with the observation made by others, that
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retroviral transduction with a regulatory gene does not influence the overall cytokine profile of the transduced cells. In addition, it is known that retroviral integration and expression of GFP does not interfere with T cell function, such as surface molecule expression, cytokine pattern, antigen specificity and cytotoxicity. IL-10-GFP CD4+ cells secreted low levels of IL-2 and IL-4, substantial levels of IL-5 and TNF-α and high levels of IFN-γ (figure 3 and data not shown). The culture conditions could have favoured the production of IFN-γ; as it is known that even skin and peripheral blood derived Th2 clones may produce IFN-γ after repeated feeder stimulations, presumably as a result of IFN-γ inducing factors like IL-12 produced by feeder cells and upregulation of IL-12R beta2 chain expression by T cells cultured in feeder mixtures. Although IFN-γ is generally regarded as a pro-inflammatory cytokine, it is also produced by regulatory T cells and it may be needed to counteract T cell mediated immune activation in the early response of intestinal inflammation. Indeed, it has recently been reported that naturally occurring regulatory T cells are characterized by increased production of both IL-10 and IFN-γ.

Systemic administration of rhIL-10 to patients with Crohn’s disease lacks tissue specificity and may have systemic side effects. Hence, for intestinal mucosal IL-10 transgene expression without systemic IL-10 exposure, IL-10-GFP transduced CD4+ cells need to specifically home to the intestinal mucosa. The integrin α4β7 regulates selective homing of lymphocytes to mucosal sites by binding to MAdCAM-1, which is selectively found in the gut-associated lymphoid tissue. A4β7 is normally present at low levels on all naïve peripheral blood lymphocytes and on a subset of memory T cells with gut tropism. MAdCAM-1 is upregulated in the inflamed intestinal mucosa, increasing the chances of T-cell homing to these areas. We therefore identified α4β7 as a candidate to specifically direct the transduced CD4+ cells to the intestine.

The expression of α4β7 on the surface of the engineered CD4+ cells was high, and we demonstrated that α4β7 did mediate specific adhesion of the transduced CD4+ cells to the mucosal addressin MAdCAM-1 in vitro. High α4β7 expression was not specific for IL-10-GFP CD4+ cells, and was also observed on control GFP and non-transduced CD4+ cells cultured in parallel. Although a recent study reported that a transduction procedure with an MMLV vector containing GFP per se did not alter the expression of
lymphocyte homing receptors, their finding is in concordance with another study that demonstrated upregulation of $\alpha 4\beta 7$ by both human and murine CD4+ cells under similar culture conditions. In addition, IL-10-GFP transduced cells showed an intermediate expression of CD62L and high expression of CD18 and CD44, other adhesion molecules involved in mucosal homing.

The concept of therapeutic use of retrovirally transduced T cells expressing IL-10 was proven in experimental ovalbumin (OVA)-induced arthritis, also a Th1 mediated disease. In this model, OVA-specific T cells infected with a retroviral vector containing IL-10 migrated to the inflamed joint, and exerted a local anti-inflammatory response. In experimental autoimmune encephalomyelitis (EAE), a Th1 mediated disease, efficient delivery to the site of inflammation of either therapeutic or exacerbating factors by genetically modified T cells has been reported. Encouraging results were obtained from a study showing that antigen specific T cell clones transfected with IL-10 cDNA were able to inhibit EAE. In these disease models the causative antigens have been identified, enabling the use of antigen-specific regulatory T cells. Neither in Crohn's disease, nor in most experimental animal models of inflammatory bowel disease, causative antigens have been identified. However, we expect that specific T cells within the large polyclonal IL-10 transduced population will be activated within the mucosal compartment and will exhibit regulatory functions including inhibition of proliferation of T cells and downregulation of IL-12 production by DCs (a mechanism known as "bystander suppression"). Indeed, we have recently demonstrated the feasibility of this approach in experimental colitis induced by transfer of CD45RBhigh CD4+ cells in SCID mice.

In conclusion, efficient transduction of peripheral blood T cells with a retroviral vector containing IL-10 is feasible and this results in long-term IL-10 production. IL-10 transduced cells actively suppress proliferation of autologous responder cells and IL-12 production by DCs. These cells have the adhesion characteristics necessary to home to the intestinal mucosa and produce IL-10 in an activation-dependent manner. A major advantage of the ability to engineer T cells ex vivo is that systemic exposure to the retroviral vector is avoided, and that administration of such cells is not expected to cause high systemic IL-10 concentrations. Therefore, we consider such ex vivo engineered
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regulatory T cells an attractive approach to maintain long-term remissions in Crohn’s disease.

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