

Dendritic cells lentivirally engineered to overexpress interleukin-10 inhibit contact hypersensitivity responses, despite their partial activation induced by transduction-associated physical stress

Verena Besche¹
Nadine Wiechmann¹
Timo Castor¹
Stefanie Trojandt¹
Yvonne Höhn¹
Hanna Kunkel²
Manuel Grez²
Stephan Grabbe¹
Angelika B. Reske-Kunz¹
Matthias Bros^{1*}

¹University Medical Center of the Johannes Gutenberg-University, Clinical Research Unit Allergology, Department of Dermatology, Mainz, Germany

²Institute for Biomedical Research, Georg-Speyer-Haus, Frankfurt/Main, Germany

*Correspondence to: Matthias Bros, Clinical Research Unit Allergology, Department of Dermatology/VFG, University Medical Center of the Johannes Gutenberg-University, Obere Zahlbacher Strasse 63, D-55131 Mainz, Germany. E-mail: mbros@uni-mainz.de

Abstract

Background Dendritic cells (DCs) constitute an attractive target for immunotherapeutic approaches. Because DCs are largely refractory to transfection with plasmid DNA, several viral transduction protocols were established. The potential side-effects of lentiviral transduction on the phenotype and activation state of DCs left unstimulated after transduction have not been assessed. There is a need to analyse these parameters as a result of the requirement of using DCs with a low activation state for therapeutic strategies intended to induce tolerance.

Methods Lentivirally-transduced bone marrow (BM)-derived DCs (LV-DCs) in comparison with mock-transduced (Mock-DCs) and untreated DCs were analysed with regard to the induction of maturation processes on the RNA, protein and functional level. BM-DCs engineered to overexpress interleukin (IL)-10 were analysed for therapeutic potential in a mouse model of allergic contact dermatitis.

Results Compared with untreated DCs, Mock-DCs and LV-DCs displayed an altered gene expression signature. Mock-DCs induced a stronger T cell proliferative response than untreated DCs. LV-DCs did not further augment the T cell proliferative response, but induced a slightly different T cell cytokine pattern compared to Mock-DCs. Accordingly, the gene promoter of the DC maturation marker fascin mediated efficient expression of the model transgene IL-10 in unstimulated-transduced BM-DCs. Nevertheless, IL-10 overexpressing BM-DCs exerted tolerogenic activity and efficiently inhibited the contact hypersensitivity response in previously hapten-sensitized mice.

Conclusions Lentiviral transduction of BM-DCs results in their partial activation. Nevertheless, the transduction of these DCs with a vector encoding the immunomodulatory cytokine IL-10 rendered them tolerogenic. Thus, lentivirally-transduced DCs expressing immunomodulatory molecules represent a promising tool for induction of tolerance. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords contact hypersensitivity; fascin promoter; gene therapy; lentiviral transduction; murine dendritic cells

Received: 13 July 2009
Revised: 10 December 2009
Accepted: 8 January 2010

Introduction

Dendritic cells (DCs) exert either a tolerogenic or immunogenic function depending on their state of activation [1]. In their immature state, DCs reside as sentinels in most tissues where they constantly probe for antigen. Under homeostatic conditions, small numbers of DCs acquire a semi-mature state and exert tolerogenic function by inducing apoptosis, anergy or even a regulatory state in interacting T cells reactive to autoantigens or harmless environmental antigens. These tolerogenic DCs as well as thymus-derived natural regulatory T cells (Treg) are essential for the maintenance of peripheral tolerance. In the course of infection, DCs are activated either directly by contact with pathogen-derived components such as lipopolysaccharide (LPS) or indirectly by contact with proinflammatory cytokines produced by other pathogen-sensing cells. Fully-activated DCs constitute the most potent antigen presenting cells (APCs) capable of efficiently priming naïve T cells [2].

On the basis of their potency to either (re)establish tolerance or to evoke strong immune responses, the potential of modified DCs to be used as vaccines for immunotherapy has been evaluated in numerous studies [3]. Genetic alteration of DCs by transfection with messenger RNA *in vitro* and plasmid DNA *in vivo* was shown to allow for processing and presentation of antigenic peptides derived from a transgene [4,5], as well as for the production of immunomodulatory molecules with an autocrine and/or paracrine effect on DC function and T cell polarization [6]. Because the use of nonviral methods to transfect DCs resulted in a low transfection efficiency [7,8], several viral transduction protocols using pseudoviral particles derived from murine Molony leukemia virus, adenovirus or lentivirus have been adopted for DCs [3]. Transduction of DC progenitors using lentiviral vectors was reported to yield efficient transduction. These transduced DCs exhibited potent antigen presenting function. Lentiviral transduction of unstimulated human monocyte-derived DCs was reported to result in partial activation of the cells compared to mock-treated DCs if very high doses of lentiviral vector were used [9]. Potential side-effects associated with lentiviral transduction of murine bone marrow (BM)-DCs have been assessed solely after LPS-mediated stimulation of the cells, yielding diverse results [10,11]. To the best of our knowledge, the potential side-effects of lentiviral transduction on the phenotype and activation state of BM-DCs left unstimulated after transduction have not been assessed up to now. There is a need to analyse these parameters in unstimulated BM-DCs after transduction as a result of the essential requirement of DCs with a low activation state for therapeutic strategies intended to induce tolerance. Along the same line of reasoning, it is preferable to use BM-DCs generated in the sole presence of granulocyte macrophage colony-stimulating factor (GM-CSF) for tolerance induction. Most studies analysing the effect of lentiviral transduction on DC

phenotype and function have used BM-DCs cultivated in the presence of GM-CSF plus interleukin (IL)-4. However, the addition of IL-4 during BM-DC generation was shown to exert a stimulating effect on the phenotype and the APC activity of BM-DCs [12–14]. Consequently, although BM-DCs differentiated solely in the presence of GM-CSF exerted tolerogenic effects in a murine transplantation model [15], BM-DCs generated in the presence of GM-CSF plus IL-4 failed so because of their enhanced CD86 expression [16].

Moreover, the issue of whether murine DCs subjected to the physical stress as experienced during harvest in preparation for viral transduction displayed an altered phenotype and APC function has not been properly addressed. On the basis of the principal finding that murine DCs were sensitive to physical stress and up-regulated both major histocompatibility complex (MHC)II and costimulatory molecules (CD80, CD86) in response to harvesting and reseeded [17], we analysed stress-induced alterations in gene expression as well as in the T cell stimulatory capacity by comparing untreated and mechanically stressed, mock-transduced BM-DC populations.

In the present study, we show that the physical stress associated with the transduction protocol as well as the transduction process itself modulated the gene expression signature of DCs in a gene-specific manner. The partial DC activation induced by physical stress was also reflected by an enhanced stimulatory capacity for allogenic T cells. Contact of DCs with lentiviral particles did not further augment their T cell stimulatory capacity, but resulted in a modified pattern of cytokines secreted by the cocultured T cells. In accordance with the elevated activation state of DCs as a result of the transduction protocol, the promoter of the DC-specific maturation marker *fascin* was able to drive efficient transgene expression in transduced DCs in the absence of LPS stimulation. Nonetheless, BM-DCs engineered to overexpress IL-10 displayed tolerogenic activity *in vitro* as well as *in vivo* in a therapeutic mouse model of allergic contact dermatitis.

Materials and methods

Mice

Female C57BL/6 and BALB/c mice were bred and maintained in the Central Animal Facilities of the University of Mainz under specific pathogen-free conditions on a standard diet. The 'Principles of Laboratory Animal Care' (NIH publication no. 85-23, revised 1985) were followed.

Cells

HEK 293T cells were cultured in DMEM (Lonza, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS low endotoxin; PAA, Cölbe, Germany), 2 mM L-glutamine (Biochrom AG, Berlin, Germany), 100 U/ml penicillin,

and 100 µg/ml streptomycin (Invitrogen Gibco, Paisley, UK) (293T medium). Cells were grown in culture flasks (175 cm³; Greiner, Nürtingen, Germany) and were split (1:100) every 5–7 days. BM-DCs were generated from bone marrow progenitors of C57BL/6 mice as reported by Scheicher *et al.* [18] with modifications as described elsewhere [19], except that DC culture supernatants were replenished on days 3 and 6, and part of the DC cultures was stimulated with LPS (1 µg/ml; Calbiochem, Schwalbach, Germany) on day 8 for 24 h to yield mature DCs (mDCs). The DC medium used was IMDM (Gibco/Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 5% of GM-CSF containing cell culture supernatant [20] (a kind gift by Dr B. Stockinger, MRC National Institute for Medical Research, London, UK). Immature BM-DCs (iDCs) harvested on day 9 of culture for experiments contained >85% CD11c⁺ cells as assessed by cytofluorometric analysis.

Vectors

The packaging plasmid pCMVΔR8.91, which harbors gag, pol, rev and tat genes derived from HIV-1 [21], and the vesicular stomatitis glycoprotein G-encoding plasmid pMD.G2 [22] were used. The HIV-1-derived vector pCDH1-EF1-copGFP was obtained from SBI (Mountain View, CA, USA). We have previously described the DC-focussed functional activity of the murine fascin gene promoter [5]. A 2.5-kb fascin gene promoter fragment (GenBank accession number AJ318756) was excised from pZero-2 (Invitrogen, Groningen, Netherlands) cloning vector backbone by *Bam*HI digest, followed by fill in reaction and subsequent *Nhe*I digest. To replace the cytomegalovirus (CMV) promoter in pCDH1-EF1-copGFP by the fascin promoter, the lentiviral vector was digested with *Spe*I, followed by Klenow treatment and *Nhe*I digest. The fascin promoter fragment was cloned into the promoterless lentiviral vector backbone, thereby obtaining pFscnH1-EF1-copGFP. cDNA derived from murine LPS-stimulated BM-DCs was used to amplify the open reading frame of IL-10 by polymerase chain reaction (PCR) employing primers harboring restriction sites (underlined) for *Nhe*I (5'-GGGCTAGCCTTGCAGAAAAGAGAGCTCCA-3') and *Bsp*II (antisense primer: 5'-GGTTCGAATGGAGTCCAGCAGACTCAAT-3'). The PCR product was digested with the aforementioned restriction enzymes, and the IL-10 mini-gene was cloned into likewise digested pCDH1-EF1-copGFP and pFscn1-EF1-copGFP vectors.

Lentivirus production

For transfection, 293T cells were plated at 3.5×10^6 cells/9 ml 293T medium into cell culture dishes (10 cm in diameter; Greiner). On the next day, the culture medium was exchanged 2–3 h prior to transfection.

293T cells were cotransfected with pCMVΔR8.91 (10 µg), pMD.G2 (4 µg) and a vector plasmid (13.2 µg) using the calcium chloride transfection procedure in the presence of chloroquine sulfate (10 µM; Sigma-Aldrich, Deisenhofen, Germany). The supernatant was removed 16 h later, and 5 ml of fresh culture medium were added. Supernatants containing lentiviral particles, harvested both 24 h and 48 h later, were pooled and stored at –70 °C. Lentiviral RNA titers were determined by real time PCR employing the Lenti-X qRT-PCR Titration Kit (Clontech, Mountain View, CA, USA).

Lentiviral transduction of BM-DCs

On day 5 of culture, immature BM-DCs were replated at 0.75×10^6 cells/0.5 ml DC medium per well (six-well tissue culture plate; Greiner). DCs were transduced on days 5 and 6 applying each 3.5×10^9 viral RNA copies/well in the presence of protamin sulfate (4 µg/ml; Sigma-Aldrich) [12] in a total volume of 2.5 ml, and plates were centrifuged (340 g for 10 min at 37 °C). DCs were washed 5 h later by replacing 1 ml of volume with fresh DC medium. After the second round of transduction, 4 ml of fresh DC medium were added in addition. Lentivirally-transduced BM-DCs were harvested on day 9 (LV-DCs). For mock transduction, DCs were treated the same way, except that supernatants of mock-transfected 293T cells cultured and treated like transfected 293T cells were used (Mock-DCs). To analyse the effects induced by the physical stress associated with the transduction procedure, DCs were treated the same way as transduced DCs, without the addition of 293T supernatant (Stress-DCs). Aliquots of cells were stimulated with LPS (1 µg/ml) on day 8 for 24 h.

Flow cytometry

BM-DCs (5×10^5) were washed in staining buffer [phosphate-buffered saline (PBS)/2% FCS]. To block Fc receptor-mediated staining, cells were incubated with rat anti-mouse CD16/CD32 antibody (2.4.G2) (Dianova, Hamburg, Germany) for 10 min on ice. Afterwards, cells were incubated with phycoerythrin-conjugated antibodies recognizing MHC class II I-A/I-E (M5/114.15.2), CD86 (GL1) (both purchased from BD Pharmingen, San Diego, CA, USA) and CD80 (16-10A1) (eBioscience, San Diego, CA, USA) for 20 min on ice. Antibodies were of rat (MHCII, CD86) or hamster (CD80) origin. Appropriate isotype controls were used. Flow cytometric analysis was performed using a FACScan flow cytometer (Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA) equipped with CellQuest Software.

Cytokine assays

Enzyme-linked immunosorbent assay (ELISA) capture antibodies for murine interferon (IFN)-γ (clone R4-6A2) and IL-5 (TRFK5) were purchased from BD

Pharmingen; antibodies for murine IL-2 (JES6-1A12), IL-4 (11B11) and IL-10 (JES052A5) were obtained from eBioscience. Biotinylated detection antibodies for murine IFN- γ (XMG1.2) and IL-5 (TRFK4) were obtained from BD Pharmingen, for murine IL-10 (BAF417) from R&D Systems (Minneapolis, MN, USA), and for murine IL-2 (JES6-5H4) and IL-4 (BVD6-24G2) from eBioscience. Antibodies were used as recommended by the manufacturer. Recombinant murine cytokines IFN- γ and IL-5 used for ELISA standards were purchased from BD Pharmingen, recombinant murine IL-10 from R&D Systems, and recombinant IL-2 and IL-4 from eBioscience. The minimal cytokine detection levels were 15 pg/ml in case of IFN- γ , IL-10, and IL-2, and 8 pg/ml in case of IL-4 and IL-5.

Real-time reverse transcriptase-PCR analysis

Total RNA was isolated from at least 5×10^5 BM-DCs by using the RNeasy MiniPlus kit (Qiagen, Hilden, Germany), which includes removal of genomic DNA, according to the manufacturer's instructions. Ten μ l of eluted RNA were reverse-transcribed applying a 1:1 mix of Oligo-dT and random hexamer primers using iScript (Bio-Rad, Munich, Germany) as recommended. Sequences of primer pairs used for detection of mRNA expression levels and real-time PCR conditions have been described previously [23]. Additional primers were used to detect expression of viral stress-associated markers IFN α 2 (sense: 5'-GCCATCCCTGTGCTGCGAGA-3', antisense: 5'-CAGCCAGCAGGGCGTCTTCC-3') and IFN β 1 (sense: 5'-ACGCCTGGATGGTGGTCCGA-3', antisense: 5'-ACCAGTCCCAGAGTCCGCCT-3'). Primers were purchased from Eurofins MWG Synthesis (Ebersberg, Germany).

Proliferation assays

BALB/c T cells were enriched from splenocytes by nylon wool adherence (>70% CD90⁺ cells as assessed by flow cytometry) as described previously [23]. T cells (3×10^5) were cocultured with graded numbers of irradiated (30 Gy) C57BL/6 BM-DCs for 4 days on flat-bottom 96-well tissue culture plates in 200 μ l of DC medium without GM-CSF. T cell proliferation was assessed by the uptake of [³H]thymidine (0.25 μ Ci/well) for the last 16 h of mixed lymphocyte reaction cultures. Cells were harvested onto glass fiber filters and retained radioactivity was measured in a liquid scintillation counter (1205 Betaplate, LKB Wallac, Turcu, Finland).

Contact hypersensitivity

Contact hypersensitivity (CHS) was induced as described previously [24] with modifications. Briefly, female C57BL/6 mice were sensitized with 17 μ l of a 0.4%

solution of dinitrofluorobenzene (DNFB) (Sigma-Aldrich) applied to the shaved abdomen on two days (days 0 and 1). Unstimulated lentivirally-transduced BM-DCs were harvested, γ -irradiated (30 Gy) and pulsed at a density of 5×10^6 /ml with 5 μ M dinitrobenzenesulfonate (DNBS; Sigma-Aldrich) for 10 min at 37 °C. The trypan blue exclusion assay confirmed viability of the cells. DCs (5×10^5 cells in 100 μ l of PBS per animal) or 100 μ l of PBS alone were injected subcutaneously on day 5 of sensitization of the mice. Seven days later, mice were challenged by applying 8.5 μ l of a 0.2% solution of DNFB to both sides of both ears. Ear thickness was assessed blindly using an engineer's micrometer (Mitutoyo, Tokyo, Japan) prior to challenge and 48 h after challenge, and ear swelling was calculated as the respective difference. Mice challenged without previous sensitization exhibited ear swelling ≤ 0.03 mm.

Statistical analysis

Data were analysed for statistically significant differences using Student's *t*-test.

Results

Physical stress associated with the lentiviral transduction procedure results in partial maturation of BM-DCs

The lentiviral transduction procedure of BM-DCs comprised two cycles of transduction on days 5 and 6 of the BM-DC generation culture. With our transduction protocol, we consistently yielded over 90% transduction efficiency in unstimulated and LPS-stimulated BM-DCs, as assessed by FACS analysis of an alternative green fluorescent protein (copGFP) encoded by the lentiviral vector pCDH1-EF1-copGFP (LV) (Figure 1A). Unstimulated and LPS-stimulated Mock-DCs, which had been subjected to the same transduction procedure, as were the LV-DCs but without application of lentiviral particles, served as a negative control. Because the transduction protocol involved cell replating, centrifugation and washing steps, we analysed whether the physical stress experienced by the DCs during the transduction procedure functions as a maturation stimulus for the DCs. To this end, we assessed the T cell allostimulatory capacity of untreated DCs and of physically stressed Mock-DCs as well as LV-DCs. As depicted in Figure 1B, untreated iDCs exhibited a moderate allostimulatory potential, which was significantly enhanced when LPS-stimulated mDCs were used as stimulators. In comparison, Mock-DCs used without stimulation, and after stimulation with LPS, respectively, induced a significantly higher T cell proliferative response than did their untreated counterparts. Contact with the lentiviral particles did not result in further activation of the DCs because LV-DCs induced comparable T cell proliferation as did Mock-DCs, regardless of whether

they were used unstimulated or after LPS stimulation (Figure 1C). The supernatant of mock-transfected 293T cells, which was used to generate Mock-DCs, might contain biological mediators with DC activating potential. On the other hand, the physical stress associated with the transduction procedure may result in activation of the DCs. We therefore compared the allostimulatory potential of Mock-DCs and of DCs that had been subjected to physical stress as associated with lentiviral transduction without the addition of supernatant of mock-transfected 293T cells (Stress-DCs) (Figure 1D). At an unstimulated state, Mock-DCs displayed a somewhat higher T cell stimulatory capacity than Stress-DCs. On the other hand, at

a stimulated state, both DC populations exerted comparable allostimulatory capacity. Thus, unstimulated and LPS-stimulated lentivirally-transduced BM-DCs exhibit an enhanced T cell stimulatory potential, which is mainly a result of the physical stress associated with the transduction procedure.

Physical stress experienced by DCs rather than their contact with lentiviral particles alters the gene expression signature of BM-DCs

To reveal whether immunorelevant genes were differentially regulated in BM-DCs subjected to physical stress and lentiviral transduction, we monitored the gene expression signatures of Mock-DCs and LV-DCs compared to untreated DCs with regard to transcripts encoding costimulatory molecules (Figure 2A), cytokines (Figure 2B) and other DC markers (Figure 2C). Compared to iDCs, unstimulated Mock-DCs and LV-DCs displayed moderately higher mRNA expression levels of the tolerogenic marker IL-1 receptor antagonist (IL-1RA) (Figure 2B), as well as the viral stress-marker IFN α 2 and the tolerogenic surface molecule mitogen-activated protein kinase phosphatase-1 (Figure 2C). On the other hand, the level of transcripts encoding the tolerogenic markers IL-1R2 and glucocorticoid induced leucine zipper (GILZ) were decreased in Mock-DCs and LV-DCs.

Stimulation of the various DC populations with LPS resulted in the enhanced expression of most transcripts encoding the costimulatory molecules, maturation markers and cytokines assessed. On the other hand, some

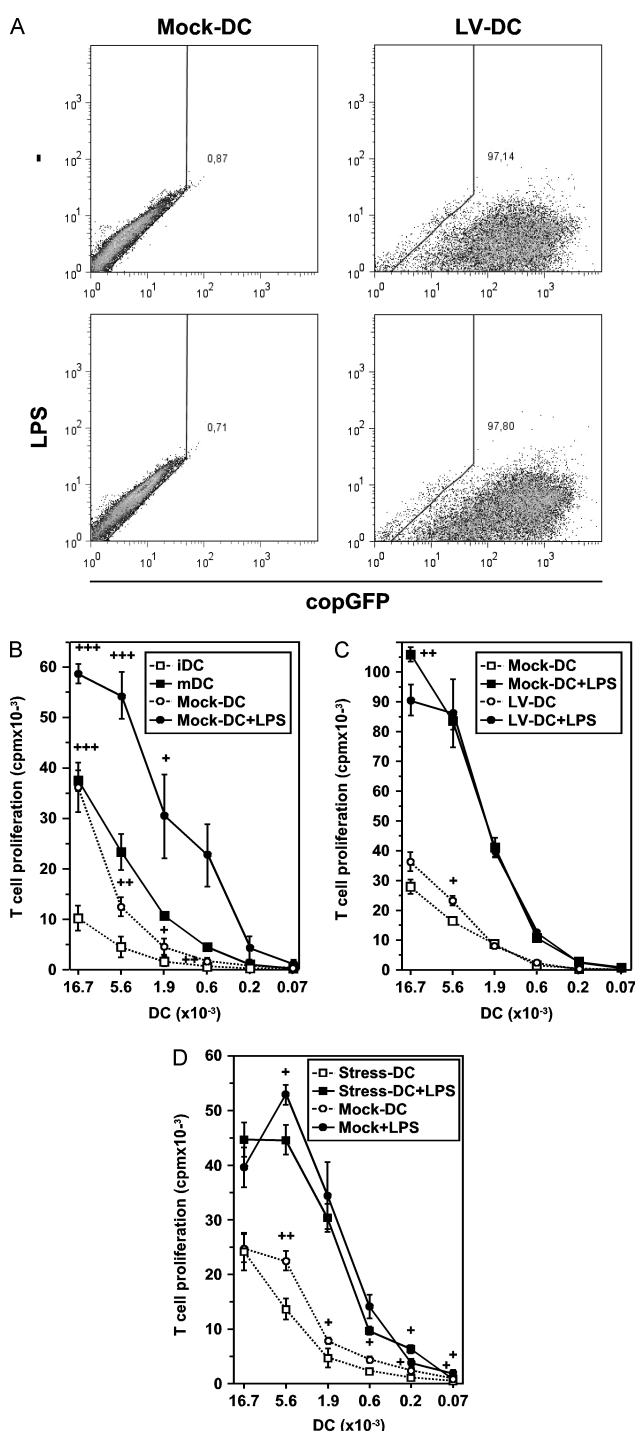
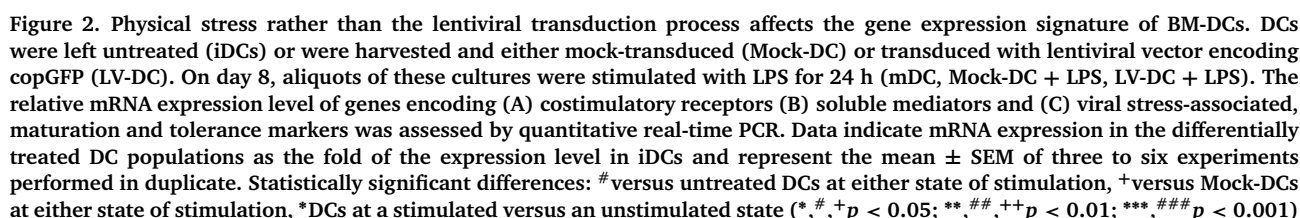


Figure 1. Physical stress as associated with the lentiviral transduction procedure enhances the T cell stimulatory capacity of BM-DCs. BM-DCs were left untreated (iDC) or were harvested and either mock-transduced without (Stress-DC) or with supernatants of mock-transfected 293T cells (Mock-DC) or were lentivirally-transduced with a vector encoding copGFP (LV-DC). On day 8, aliquots of these cultures were stimulated with LPS for 24 h (mDC, Mock-DC + LPS, LV-DC + LPS, Stress-DC + LPS). (A) Transduction efficiencies were assessed by cytofluorometric analysis for copGFP expression. Graphs are representative of six independent experiments. (B–D) DCs were irradiated and cocultured in titrated numbers with 3×10^5 allogenic BALB/c T cells for 4 days in 0.2 ml of culture medium. T cell proliferation was assessed as uptake of [^{3} H]thymidine for the final 16 h of culture. Data indicate the mean $\pm$ SEM of triplicate cultures and are representative of three (B), four (C) or two (D) independent experiments. Statistically significant differences: (B) Untreated DCs and Mock-DCs showed statistically significant differences at a stimulated versus an unstimulated state at each data point ($p < 0.05$). +Mock-DCs versus untreated DCs at either state of activation (+ $p < 0.05$; **, + $p < 0.01$; +++ $p < 0.001$). (C) Mock-DCs and LV-DCs showed statistically significant differences at a stimulated versus an unstimulated state at each data point ($p < 0.05$). +LV-DCs versus Mock-DCs at either state of activation (+ $p < 0.05$; ++ $p < 0.01$). (C) Stress-DCs and Mock-DCs showed statistically significant differences at a stimulated versus an unstimulated state at each data point ($p < 0.05$). +Mock-DCs versus Stress-DCs at either state of activation ($p < 0.05$)



absence of LPS stimulation, both Mock-DCs and LV-DCs expressed MHCII and the costimulatory molecules CD80 and CD86 at a comparable level (Figure 3). After stimulation with LPS, the surface expression of the three markers was up-regulated in both DC populations, although to a varying extent. (Figure 3). Thus, lentiviral transduction modifies LPS-induced up-regulation of the surface molecules tested.

Physically stressed and lentivirally-transduced BM-DCs induce a slightly different pattern of T cell cytokines

Next, we analysed whether the DC populations differed in their T cell polarizing properties. In cocultures of C57BL/6 BM-DCs and allogenic BALB/c T cells, both unstimulated

Effect of lentiviral transduction on DCs

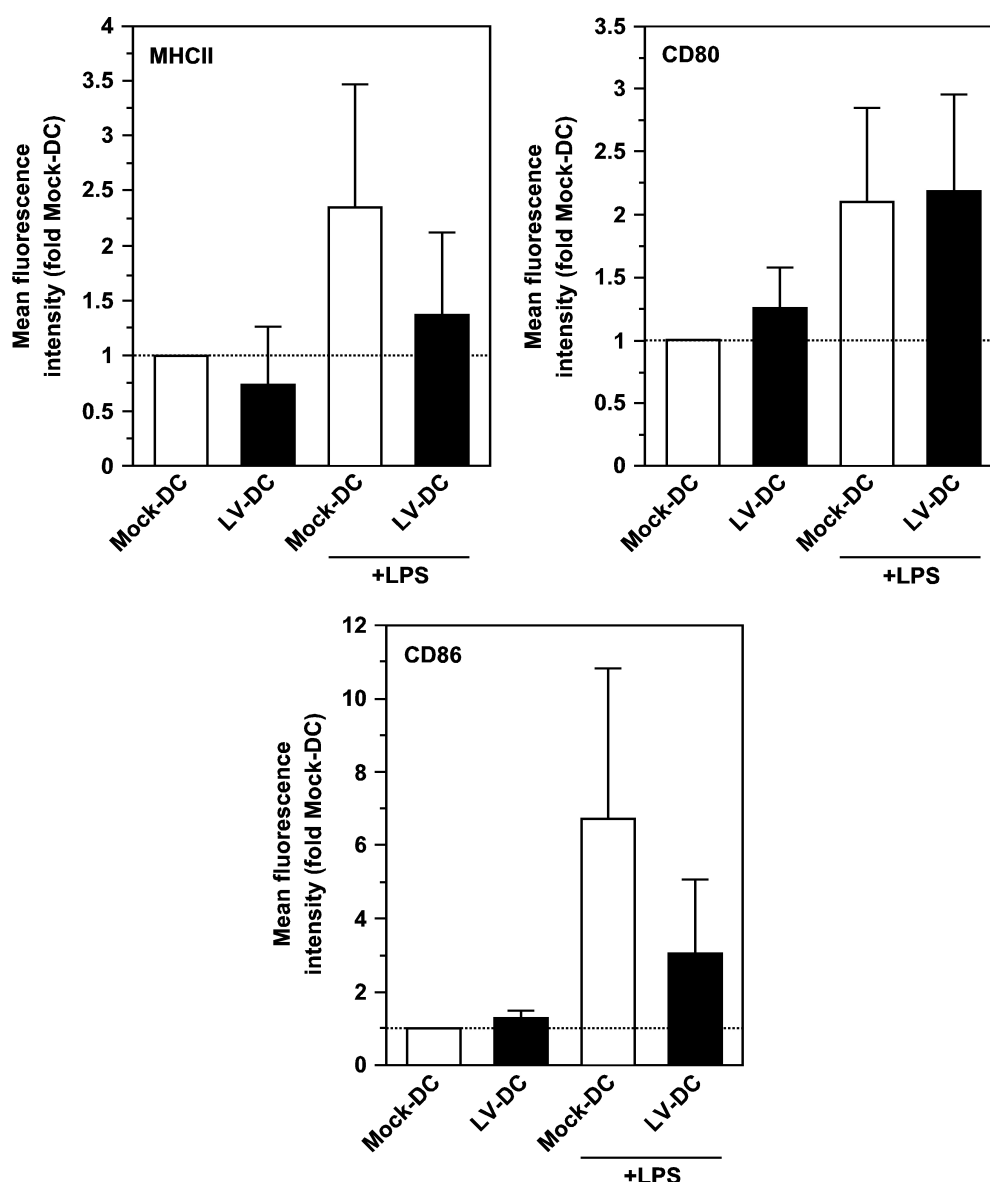


Figure 3. Lentiviral transduction affects the cell surface phenotype of murine BM-DCs. DCs were mock-transduced (Mock-DC) or transduced with the lentiviral vector encoding copGFP (LV-DC), and aliquots of cultures were stimulated with LPS on day 8 for 24 h. Differentially treated DCs at an unstimulated and a LPS stimulated state were stained with monoclonal antibodies specific for MHCII and costimulatory molecules (CD80, CD86). Mean fluorescence intensities (MFI) represent the mean $\pm$ SEM of two to three independent experiments and were normalized to the MFI of unstimulated Mock-DCs (MHCII: 32.77 ± 13.28 , CD80: 46.64 ± 17.29 , CD86: 14.80 ± 4.39) arbitrarily set to one

Mock-DCs and LV-DCs induced the production of the Th1 marker cytokine IFN- γ at comparable levels (Figure 4A). However, after stimulation with LPS, Mock-DCs in contrast to LV-DCs induced the enhanced production of IFN- γ by T cells, although this was not statistically significant. Production of the Th2 marker cytokines IL-4 and IL-5 as well as of the Th2/Treg marker cytokine IL-10 was very low, and therefore may lack biological significance. Although unstimulated Mock-DCs and LV-DCs did not significantly differ in the induction of IL-4 production (Figure 4B) and also evoked a comparable secretion of IL-5 (Figure 4C), stimulated LV-DCs induced significantly reduced IL-4 production compared to Mock-DCs, but significantly enhanced

IL-5 production. Mock-DCs and LV-DCs induced the production of comparable amounts of IL-10 and IL-2 at either state of activation (Figures 4D and 4E). Similar to the allostimulatory model, unstimulated and LPS-stimulated mock-transduced and lentivirally-transduced BM-DCs pulsed with MOG35-55 peptide induced slightly different cytokine patterns in cocultured MOG-reactive 2D2 T cells, but provoked a comparable T cell proliferative response (data not shown). Taken together, the phenotypic differences observed between LPS-stimulated Mock-DCs and LV-DCs have no impact on their potential to induce T cell proliferation, but slightly affect the pattern of cytokines produced by the interacting T cells.

The murine DC-focussed fascin gene promoter facilitates transgene expression in BM-DCs irrespective of their state of activation in the absence of LPS stimulation

As further evidence for partial activation of BM-DCs by the physical stress associated with the lentiviral transduction procedure, we assessed the capacity of the fascin promoter to drive expression of IL-10 in transduced unstimulated DCs compared to LPS-stimulated DCs. We have previously shown that expression of the actin bundling protein fascin in DCs correlates with their state of activation and, as a result of its restricted expression pattern, constitutes a DC maturation marker both in mouse [25] and man [26]. In the mouse, a 2.5-kb 5'-flanking sequence stretch of the first exon of the murine fascin gene acts as a DC-specific promoter that facilitates transgene expression in maturing and mature, but not in immature DCs [5].

Transduction of BM-DCs with IL-10 encoding lentivirus under expressional control of either the DC-specific cellular fascin gene promoter (pFscnH1-IL-10-EF1-copGFP) or the viral ubiquitously active CMV promoter (pCDH1-IL-10-EF1-copGFP) used for comparison (Figure 5A) yielded similar rates of copGFP+ cells (Figure 5B), which were comparable to the rates noted for the empty control vector (LV) (Figure 1A). As depicted in Figure 5C, the fascin promoter mediated IL-10 production in unstimulated and LPS-stimulated DCs to a comparable extent. This observation suggests that lentiviral transduction results in the extensive activation of transcription factors mediating fascin promoter activity. As expected, the strong viral CMV promoter was more efficient than the cellular fascin promoter in mediating the production of the cytokine.

IL-10 overexpressing BM-DCs display a protolerogenic phenotype and are poor T cell stimulators

On the basis of our finding of partial activation of BM-DCs subjected to physical stress as associated with lentiviral transduction, we investigated whether lentivirally mediated overexpression of the potent anti-inflammatory cytokine IL-10 was able to counteract this effect and re-establish a tolerogenic BM-DC state. In real-time PCR analysis, unstimulated DCs transduced with pCDH1-IL10-EF1-copGFP expressed mRNA species encoding costimulatory receptors at a similar (CD86) or at a lower (CD80, OX40 l) level than LV-DCs (Figure 6A, left). Upon stimulation, expression of the monitored mRNA species was up-regulated in LV-DCs. By contrast, DCs overexpressing IL-10 were largely refractory to stimulation-induced up-regulation of either molecule. With regard to cytokines, we noted unaltered (IL-6) or lower (IL-12p40) mRNA expression in unstimulated IL-10 overexpressing DCs compared to LV-DCs (Figure 6A, right). On the other hand, IL-10 overexpressing DCs displayed a higher IL-10 mRNA

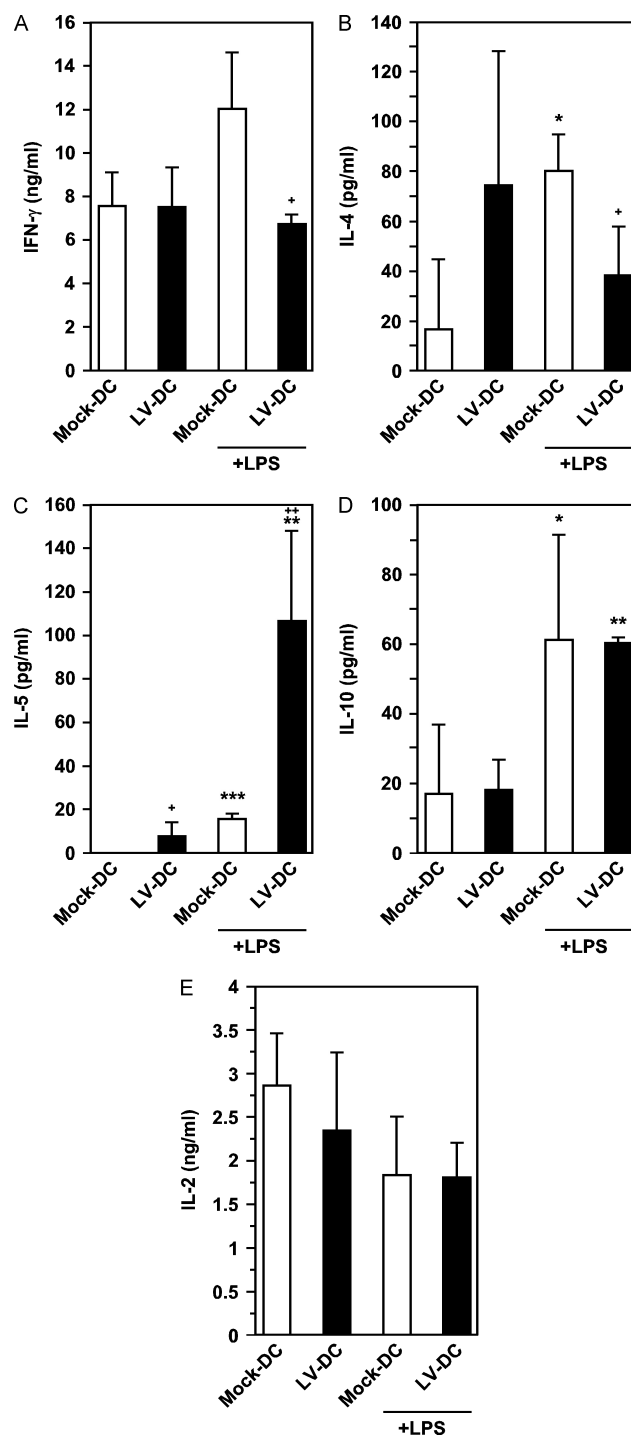


Figure 4. Lentiviral transduction of BM-DCs affects the cytokine pattern of cocultured allogeneic T cells. Mock-DCs and LV-DCs at an unstimulated or a LPS-stimulated state (0.75×10^5 cells/ml) were cocultured with 3×10^6 ml BALB/c T cells. Culture supernatants were harvested on day 3 of coculture and assayed by ELISA for contents of (A) IFN- γ , (B) IL-4, (C) IL-5, (D) IL-10 and (E) IL-2. Data represent the mean $\pm$ SEM of three to six experiments. Statistically significant differences: *DCs at stimulated versus unstimulated state, +LV-DCs three to six Mock-DCs at either state of activation (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

level than LV-DCs, in accordance with increased IL-10 production (Figure 5C). In response to stimulation,

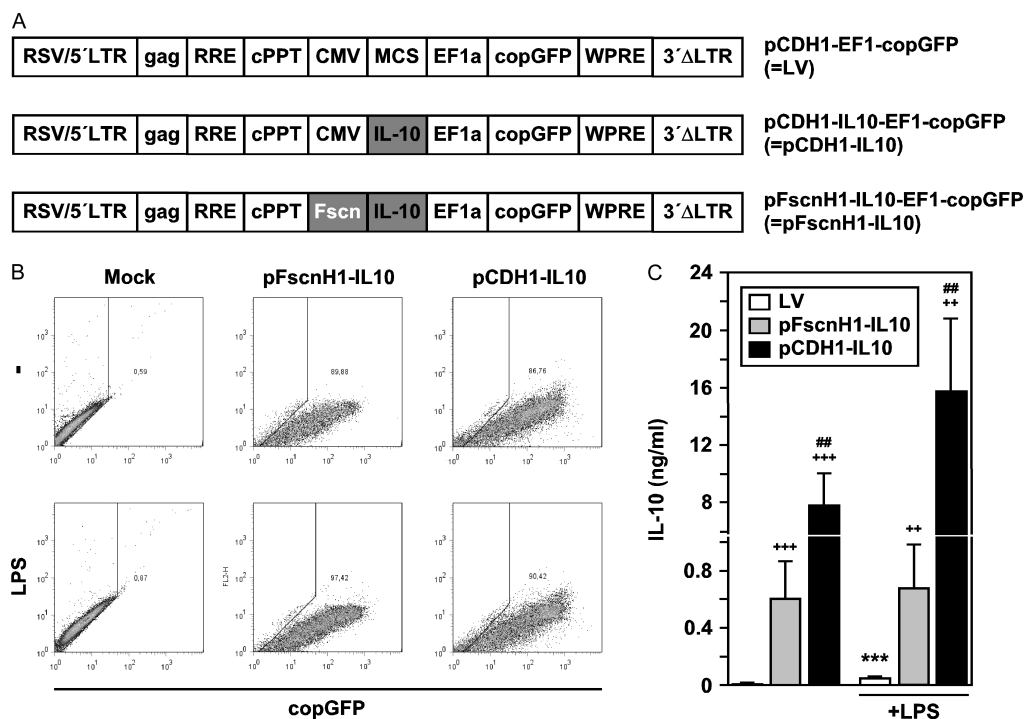


Figure 5. The DC-specific fascin gene promoter drives transgene expression in transduced BM-DCs at an unstimulated and a LPS-stimulated state to a comparable extent. (A) Structure of the lentiviral vectors used. Lentiviral vector pCDH1-EF1-copGFP was used as a control vector and for cloning (see Materials and Methods). RSV/5' LTR, chimeric RSV promoter/U5 long-terminal repeat of HIV-1; gag, packaging signal; RRE, Rev response element; cPPT, central polypurine tract; CMV, human immediate early cytomegalovirus promoter; MCS, multiple cloning site; EF1 α , human elongation factor 1 alpha promoter; copGFP, copepod green fluorescent protein; WPRES, woodchuck hepatitis virus post-transcriptional regulatory element; 3' Δ LTR, self-inactivating 3' LTR with deletion in the U3 region; Fscn, fascin promoter. (B, C) BM-DCs were either mock-transduced (Mock) or were transduced with the lentiviral control vector (LV) or IL-10 encoding expression vectors under transcriptional control of the fascin (pFscnH1-IL10) or the CMV (pCDH1-IL10) promoter and were harvested on day 9. Part of the cultures had been stimulated with LPS for the last 24 h of culture (B) Transduction efficiencies were assessed by cytofluorometric analysis for copGFP expression. Graphs are representative of three independent experiments. (C) Cell culture supernatants of lentivirally-transduced DCs were assessed for IL-10 content by ELISA. Data represent the mean $\pm$ SEM of three independent experiments. Statistically significant differences: *versus unstimulated LV-DCs, +versus LV-DCs and #versus pFscnH1-IL10-DCs, each at either state of activation (++, ## $p < 0.01$; ***, +++ $p < 0.001$)

LV-DCs up-regulated the expression of either cytokine mRNA species monitored. However, IL-10 overexpressing DCs displayed an attenuated up-regulation of IL-6 and IL-12p40 mRNA, whereas IL-10 mRNA expression was enhanced.

Unstimulated IL10 overexpressing DCs and LV-DCs expressed comparable surface levels of CD86 (Figure 6B). LV-DCs responded to LPS-mediated stimulation with elevated surface expression, which was much less pronounced in IL10 overexpressing DCs. In accordance with their protolerogenic phenotype, IL-10 overexpressing DCs displayed a significantly lower allogenic T cell stimulatory capacity than LV-DCs both at an unstimulated state and after stimulation with LPS (Figure 6C).

BM-DCs engineered to overexpress IL-10 exert tolerogenic activity in CHS

The finding in the present study of a protolerogenic phenotype and poor T cell stimulatory capacity of IL-10 overexpressing DCs prompted us to analyse their tolerogenic potency *in vivo*. To this end, we assessed their therapeutic efficacy in a mouse model of allergic contact

dermatitis. BM-DCs were transduced with lentiviral particles harboring the empty vector pCDH1-EF1-copGFP (LV) or the IL-10 encoding expression unit pCDH1-IL10-EF1-copGFP. Transduced BM-DCs were pulsed with the hapten DNBS and were injected into DNFB-sensitized mice. After subsequent challenge with DNFB on both sides of the ears, ear swelling responses were monitored 48 h later. Compared to PBS-injected control animals, mice that had received hapten-pulsed LV-DCs displayed an unaltered ear swelling response, whereas mice which had received IL-10 overexpressing BM-DCs showed a significantly diminished ear swelling response (Figure 7).

Discussion

DCs orchestrate immune responses by virtue of their ability to induce immune reactions or tolerance towards an antigen. Therefore, in numerous studies, DCs are modified for their use as vaccines for immunotherapy. In immunotherapeutic situations, which aim at inhibiting ongoing immune responses, as is the case for the treatment of autoimmunity, allergy and transplant

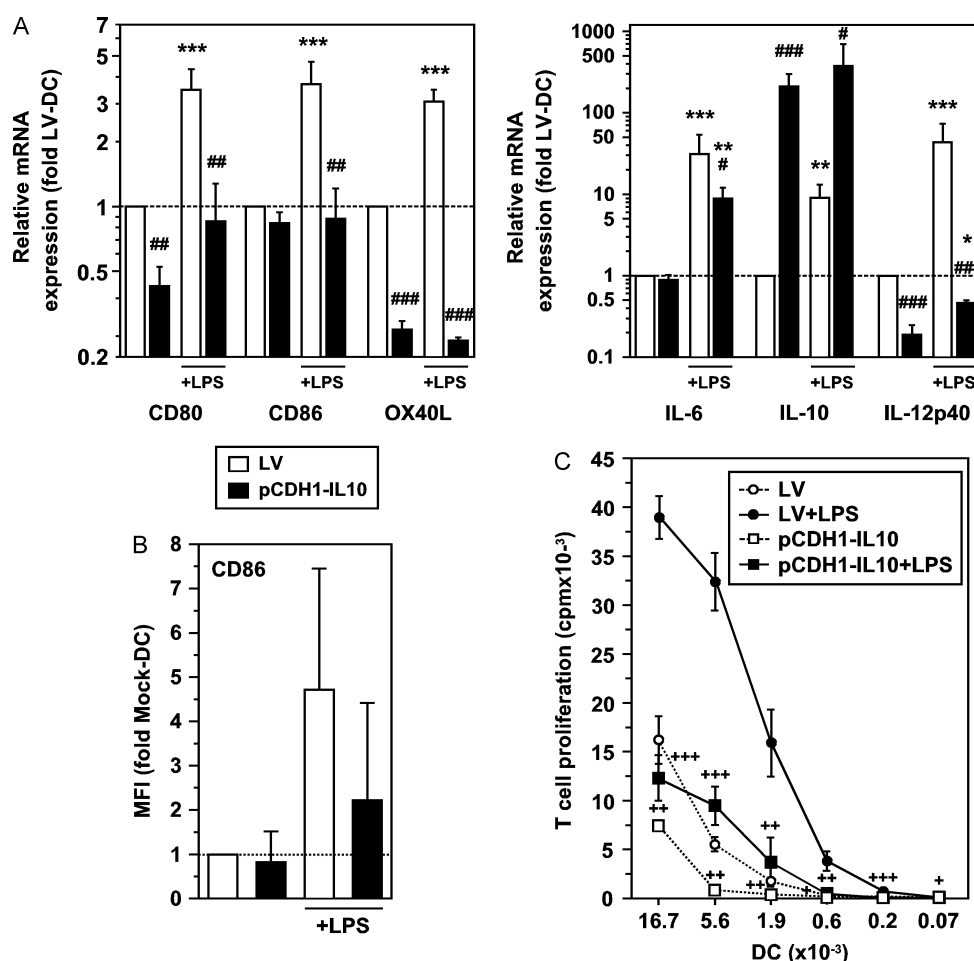


Figure 6. BM-DCs engineered to overexpress IL-10 display a tolerogenic phenotype and exert poor APC activity. DCs were transduced with the empty lentiviral vector pCDH1-EF1-copGFP (LV) or the IL-10 encoding expression vector pCDH1-IL10-EF1-copGFP (pCDH1-IL10) and aliquots of cultures were stimulated with LPS on day 8 for 24 h. (A) The relative mRNA expression level of genes encoding costimulatory receptors and soluble mediators in LV-DCs and pCDH1-IL10-DCs at an unstimulated and a LPS-stimulated state was assessed by quantitative real-time PCR. Data are given as the fold of the expression level in LV-DCs and represent the mean $\pm$ SEM of two to three experiments performed in duplicate. Statistically significant differences: # versus LV-DCs at corresponding state of stimulation, *DCs at stimulated versus unstimulated state (*, # $p < 0.05$; **, ## $p < 0.01$; ***, ### $p < 0.001$). (B) Differentially treated DCs at unstimulated and LPS-stimulated state were stained with specific anti-CD86 mAb. MFI represent the mean $\pm$ SEM of two independent experiments and were normalized to the MFI of unstimulated LV-DCs (16.69 ± 6.68) arbitrarily set to one. (C) DCs were irradiated and cocultured in titrated numbers with 3×10^5 allogenic BALB/c T cells for 4 days in 0.2 ml of culture medium. T cell proliferation was assessed as described (Figure 1). Data indicate the mean $\pm$ SEM of triplicate cultures and are representative of three independent experiments. DCs showed statistically significant differences at a stimulated versus an unstimulated state at each (LV-DCs) or the first four (pCDH1-IL10-DCs) data points ($p < 0.05$). +pCDH1-IL10-DCs versus LV-DCs at either state of activation (+ $p < 0.05$; ++ $p < 0.01$; +++ $p < 0.001$)

rejection, the use of unstimulated non-immunogenic DCs is mandatory. The process of genetic modification of DCs bears the risk of being associated with physical stress. Physical stress was previously shown to result in partial activation of BM-DCs [17]. Mechanical stress may trigger intracellular signaling pathways via the disruption of adherens junctions between adjacent cells, via altered interaction of adhesion systems with the cytoskeleton, and by differential regulation of mechanosensitive ion channels [27]. By monitoring a panel of costimulatory molecules as well as maturation and tolerance markers and viral stress-associated markers (IFN α 2, IFN β 1) we detected differential gene expression in physically stressed Mock-DCs compared to BM-DCs not subjected to physical stress, whether the DCs were used unstimulated or after

stimulation with LPS. At either state of activation, Mock-DCs also induced an increased proliferative response of allogenic T cells compared to their untreated BM-DC counterparts. The finding of stress-associated alterations of the gene expression signature of BM-DCs and concomitantly of partial activation of BM-DCs on the functional level extends the findings of Gallucci *et al.* [17], who reported on stress-induced up-regulation of MHCII and costimulatory molecule surface expression. In this regard, it is interesting that transcripts of the viral stress-associated markers IFN α 2 and IFN β 1 [28] were significantly up-regulated in Mock-DCs compared to control DCs either at both states of activation (IFN α 2) or at a stimulated state only (IFN β 1), which indicates that physical stress is sufficient to enhance mRNA expression

of both markers in BM-DCs. These results are of general importance because virtually any *ex situ* treatment of DCs may be associated with mechanical stress.

Besides the issue of stress effects associated with the transduction procedure on the phenotype and function of DCs, we investigated whether contact with the lentiviral particles altered the properties of BM-DCs. To the best of our knowledge, comparative analysis of the phenotype and APC activity of mock- and lentivirally-transduced DCs has not been performed with unstimulated BM-DCs so far. In the present study, unstimulated Mock-DCs and LV-DCs displayed no major differences with regard to surface expression of MHCII, CD80 and CD86. The same held true for gene expression of a panel of further costimulatory molecules, pro- and anti-inflammatory cytokines, tolerance markers and other molecules involved in DC/T cell interaction. Accordingly, Mock-DCs and LV-DCs exerted comparable T cell stimulatory capacity. These results suggest that the partial maturation of BM-DCs observed after lentiviral transduction is mainly a result of the physical stress associated with the transduction procedure. However, the finding of minor effects of supernatants of the virus producer cell line 293T after mock-transfection on the T cell stimulatory capacity of physically stressed DCs suggests that these supernatants may contain at least one factor that synergizes with DC-activating stimuli.

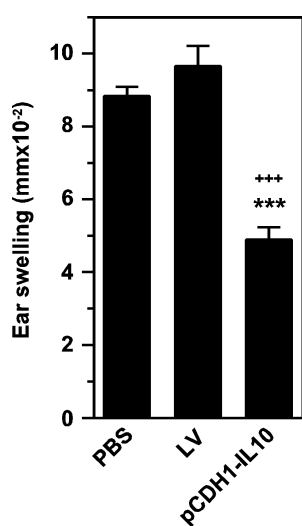


Figure 7. BM-DCs overexpressing IL-10 suppress CHS reactions. Mice were sensitized twice (days 0 and 1) with 17 μ l of 0.4% DNFB on the shaved abdomen. BM-DCs were lentivirally-transduced with the lentiviral control vector (LV) or a vector encoding IL-10 (pCDH1-IL10) as shown in Figure 5. BM-DCs were harvested, γ -irradiated, and pulsed with 5 μ M DNBS. Hapten-pulsed BM-DCs (5×10^5 cells per animal) or PBS as vehicle control were injected subcutaneously on day 5 of sensitization of the mice. Seven days later, mice were challenged with 8.5 μ l of 0.2% DNFB on each side of both ears, and the increase in ear thickness was recorded at 48 h after challenge. Data represent the mean $\pm$ SEM swelling values of five mice per group and are representative of two independent experiments. Statistically significant differences: *versus. control mice (PBS injected), + versus mice injected with LV-transduced BM-DCs (***, $+++p < 0.001$)

In response to stimulation with LPS, LV-DCs showed minor differences with regard to the surface expression of MHCII and CD86 compared to Mock-DCs. In a study by He *et al.* [11], both mock- and lentivirally-transduced murine BM-DCs used at a stimulated state expressed MHCII and costimulatory molecules to a comparable extent. However, this difference in results may be explained by the different modalities in BM-DC culture. He *et al.* [11] generated BM-DCs in the presence of IL-4, which strongly increases the surface expression of MHCII and costimulatory molecules (CD40, CD80, CD86) in unstimulated BM-DCs [12–14] and therefore may mask the side-effects associated with lentiviral transduction as apparent in BM-DCs differentiated in the presence of GM-CSF only. The data obtained in the present study are in accordance with findings reported by Breckpot *et al.* [10] who noted the higher expression of CD80 in lentivirally-transduced than in mock-transduced BM-DCs generated in the presence of GM-CSF only.

Despite subtle phenotypic differences between Mock-DCs and LV-DCs, especially when used stimulated, both DC populations exhibited a comparable capacity to induce T cell proliferation. The finding of comparable T cell stimulatory capacity of the stimulated BM-DC populations is in accordance with a previous study [10]. The content of the Th1 marker cytokine IFN- γ was at a high level in supernatants of the various allogenic DC/T cell cocultures. Similarly, when employing BM-DCs generated in the presence of GM-CSF plus IL-4, both mock and lentivirally transduced DC populations stimulated with LPS induced secretion of IFN- γ by alloreactive T cells at a comparable level [11]. We also noted comparable IL-2 levels in either DC/T cell coculture.

Production of Th2 marker cytokines (IL-4, IL-5) and of the Th2/Treg cytokine IL-10 was apparent only at a low level.

As a further indication that the procedure of lentiviral transduction induces partial activation of BM-DCs, we lentivirally-transduced BM-DCs with a vector harboring the DC-focussed promoter of the gene encoding the actin bundling protein fascin. DCs are the only haematopoietic cells to express fascin [29]. Expression of fascin in DCs correlates in strength with the degree of DC activation [26,27]. We have previously shown that the 2.5-kb promoter of the fascin gene is able to drive DC-focussed expression of transgenes after gene gun-mediated immunization and to elicit potent immune responses [5,30]. As shown in the present study, lentiviral vectors harboring expression units for IL-10 under the transcriptional control of either the DC-specific fascin gene promoter or the ubiquitously active CMV promoter yielded high-level transduction rates, thereby ruling out interference of either promoter with the copGFP-driving EF1alpha promoter, as observed to occur with other promoter combinations [31]. The fascin gene promoter facilitated comparable IL-10 expression levels in transduced unstimulated and LPS-stimulated BM-DCs. This finding suggests that lentiviral transduction by itself resulted in the expression/activation of transcription

factors that are essential for fascin promoter activity. In this respect, additional stimulation with LPS does not further increase the overall activity of transcription factors controlling the fascin promoter. The lower expression level of IL-10 under the control of the fascin promoter compared to the strong viral CMV promoter in DCs is in line with earlier findings by Morita *et al.* [32], comparing the activity of the dectin-2 promoter with the activity of the SV40 promoter after transfection of cells of a DC line. The enhanced level of IL-10 produced by LPS-stimulated BM-DCs transduced with the lentiviral vector under CMV promoter control compared to their unstimulated counterparts may be a result of nuclear factor κ B responsive elements present in repetitive alignment in the CMV promoter. This is not the case with the fascin promoter. The data obtained in the present study demonstrate the suitability of the fascin promoter to drive transgene expression in BM-DCs upon lentiviral transduction. Thus, the fascin promoter might prove valuable for lentiviral transduction specifically of DCs *in vivo*.

The finding of enhanced T cell stimulatory capacity of BM-DCs subjected to physical stress raised the question of whether such DCs used at an unstimulated state are able to exert tolerogenic function, when transduced with a lentiviral vector encoding an immunomodulatory molecule. As a result of the characteristics of IL-10, which is known to induce a tolerogenic state in DCs [33] and a regulatory phenotype in T cells [34], we investigated whether BM-DCs engineered to overexpress IL-10 induce tolerance *in vivo*. Similar to previous findings [35], BM-DCs overexpressing IL-10 displayed a protolerogenic phenotype, which was largely resistant to maturation. Accordingly, these BM-DC populations exerted significantly lower primary allogenic T cell stimulatory capacity than LV-DCs at an unstimulated state and after stimulation with LPS. We chose CHS as an appropriate disease model to assess the tolerogenic capacity of IL-10 overexpressing BM-DCs because Dai and Streilein [36] have shown that IL-10-pretreated epidermal Langerhans cells, pulsed with DNBS and injected into mice, were unable to evoke sensitization. Moreover, when injected prior to sensitization with topically applied DNFB, IL-10-pretreated Langerhans cells impaired the ear swelling response. In our therapeutic setting, unstimulated hapten-pulsed IL-10 overproducing BM-DCs applied on day 5 after sensitization of mice were able to significantly impair ear swelling responses induced by challenge with the hapten compared to empty vector-transduced BM-DCs and with vehicle. Thus, lentivirally-transduced DCs expressing immunomodulatory molecules represent a promising tool for induction of tolerance.

Acknowledgements

This work was performed in partial fulfillment of the requirements of the doctoral thesis of Verena Besche and Timo Castor.

This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 548, project B5, and the Stiftung Rheinland-Pfalz für Innovation (15212-386261/761).

References

1. Tang Q, Bluestone JA. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol* 2008; **9**: 239–244.
2. Adams S, O'Neill DW, Bhardwaj N. Recent advances in dendritic cell biology. *J Clin Immunol* 2005; **25**: 87–98.
3. Breckpot K, Aerts JL, Thielemans K. Lentiviral vectors for cancer immunotherapy: transforming infectious particles into therapeutics. *Gene Ther* 2007; **14**: 847–862.
4. Figdor CG, de Vries IJ, Lesterhuis WJ, Melief CJ. Dendritic cell immunotherapy: mapping the way. *Nat Med* 2004; **10**: 475–80.
5. Ross R, Sudowe S, Beisner J, *et al.* Transcriptional targeting of dendritic cells for gene therapy using the promoter of the cytoskeletal protein fascin. *Gene Ther* 2003; **10**: 1035–1040.
6. Kikuchi T. Genetically modified dendritic cells for therapeutic immunity. *Tohoku J Exp Med* 2006; **208**: 1–8.
7. Breckpot K, Heirman C, Neyns B, Thielemans K. Exploiting dendritic cells for cancer immunotherapy: genetic modification of dendritic cells. *J Gene Med* 2004; **6**: 1175–1188.
8. Bros M, Wiechmann N, Besche V, Castor T, Sudowe S, Reske-Kunz AB. A plasmid DNA elec-troporation method allows transfection of murine DC. *J Immunol Meth* 2009; **343**: 13–20.
9. Breckpot K, Emeagi P, Dullaers M, Michiels A, Heirman C, Thielemans K. Activation of immature monocyte-derived dendritic cells after transduction with high doses of lentiviral vectors. *Hum Gene Ther* 2007; **18**: 536–546.
10. Breckpot K, Dullaers M, Bonehill A, *et al.* Lentivirally transduced dendritic cells as a tool for cancer immunotherapy. *J Gene Med* 2003; **5**: 654–667.
11. He Y, Zhang J, Mi Z, Robbins P, Falo LD. Immunization with lentiviral vector-transduced dendritic cells induces strong and long-lasting T cell responses and therapeutic immunity. *J Immunol* 2005; **174**: 3808–3817.
12. Fresnay S, Chalmers DE, Ferrand C, *et al.* Polybrene and interleukin-4: two opposing factors for retroviral transduction of bone-marrow-derived dendritic cells. *J Gene Med* 2002; **4**: 601–612.
13. Feili-Hariri M, Falkner DH, Morel PA. Polarization of naive T cells into Th1 or Th2 by distinct cytokine-driven murine dendritic cell populations: implications for immunotherapy. *J Leukoc Biol* 2005; **78**: 656–664.
14. Maroof A, Penny M, Kingston R, *et al.* Interleukin-4 can induce interleukin-4 production in dendritic cells. *Immunology* 2006; **117**: 271–279.
15. Lu L, McCaslin D, Starzl TE, Thomson AW. Mouse bone marrow-derived dendritic cell progenitors (NLDC 145⁺, MHC class II⁺, B7-1^{dim}, B7-2⁻) induce alloantigen-specific hyporesponsiveness in murine T lymphocytes. *Transplantation* 1995; **60**: 1539–1545.
16. Fu F, Li Y, Qian S, *et al.* Costimulatory molecule-deficient progenitors (MHC class II⁺, CD80^{dim}, CD86⁻) prolong cardiac allograft survival in nonimmunosuppressed recipients. *Transplantation* 1996; **62**: 659–665.
17. Gallucci S, Lolkema M, Matzinger P. Natural adjuvants: endogenous activators of dendritic cells. *Nat Med* 1999; **5**: 1249–1255.
18. Scheicher C, Mehlig M, Zecher R, Reske K. Dendritic cells from mouse bone marrow: in vitro differentiation using low doses of recombinant granulocyte-macrophage colony-stimulating factor. *J Immunol Meth* 1992; **154**: 253–264.
19. Gisch K, Gehrke N, Bros M, *et al.* Formalin-fixed *Staphylococcus aureus* particles prevent allergic sensitization in a murine model of type I allergy. *Int Arch Allergy Immunol* 2007; **144**: 183–196.
20. Zal T, Volkmann A, Stockinger B. Mechanisms of tolerance induction in major histocompatibility complex class II-restricted T cells specific for a blood-borne self-antigen. *J Exp Med* 1994; **180**: 2089–2099.
21. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat Biotechnol* 1997; **15**: 871–875.

22. Follenzi A, Sabatino G, Lombardo A, Boccaccio C, Naldini L. Efficient gene delivery and targeted expression to hepatocytes in vivo by improved lentiviral vectors. *Hum Gene Ther* 2002; **13**: 243–260.
23. Bros M, Jährling F, Renzing A, *et al.* A newly established murine immature dendritic cell line can be differentiated into a mature state, but exerts tolerogenic function upon maturation in the presence of glucocorticoid. *Blood* 2007; **109**: 3820–3829.
24. Weigmann B, Jarman ER, Sudowe S, Bros M, Knop J, Reske-Kunz AB. Induction of regulatory T cells by leflunomide in a murine model of contact allergen sensitivity. *J Invest Dermatol* 2006; **126**: 1524–33.
25. Ross R, Ross XL, Schwing J, Längin T, Reske-Kunz AB. The actin-bundling protein fascin is involved in the formation of dendritic processes in maturing epidermal Langerhans cells. *J Immunol* 1998; **160**: 3776–3782.
26. Ross R, Jonuleit H, Bros M, *et al.* Expression of the actin-bundling protein fascin in cultured human dendritic cells correlates with dendritic morphology and cell differentiation. *J Invest Dermatol* 2000; **115**: 658–663.
27. Janmey PA, McCulloch CA. Cell mechanics: integrating cell responses to mechanical stimuli. *Annu Rev Biomed Eng* 2007; **9**: 1–34.
28. Tan PH, Beutelspacher SC, Xue SA, *et al.* Modulation of human dendritic-cell function following transduction with viral vectors: implications for gene therapy. *Blood* 2005; **105**: 3824–3832.
29. Kureishy N, Sapountzi V, Prag S, Anilkumar N, Adams JC. Fascins, and their roles in cell structure and function. *BioEssays* 2002; **24**: 350–361.
30. Sudowe S, Ludwig-Portugall I, Montermann E, Ross R, Reske-Kunz AB. Transcriptional targeting of dendritic cells in gene gun-mediated DNA immunization favors the induction of type 1 immune responses. *Mol Ther* 2003; **8**: 567–575.
31. Shearwin KE, Callen BP, Egan JB. Transcriptional interference – a crash course. *Trends Genet* 2005; **21**: 338–345.
32. Morita A, Ariizumi K, Ritter R, *et al.* Development of a Langerhans cell-targeted gene therapy format using a dendritic cell-specific promoter. *Gene Ther* 2001; **8**: 1729–1737.
33. Steinbrink K, Graulich E, Kubisch S, Knop J, Enk AH. CD4⁺ and CD8⁺ anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. *Blood* 2002; **99**: 2468–2476.
34. Smits HH, de Jong EC, Wierenga EA, Kapsenberg ML. Different faces of regulatory DCs in homeostasis and immunity. *Trends Immunol* 2005; **26**: 123–129.
35. Takayama T, Tahara H, Thomson AW. Differential effects of myeloid dendritic cells retrovirally transduced to express mammalian or viral interleukin-10 on cytotoxic T lymphocyte and natural killer cell functions and resistance to tumor growth. *Transplantation* 2001; **71**: 1334–1340.
36. Dai R, Streilein JW. Ultraviolet B-exposed and soluble factor pre-incubated epidermal Langerhans cells fail to induce contact hypersensitivity and promote DNP-specific tolerance. *J Invest Dermatol* 1997; **108**: 721–726.