Enforced IL-10 Expression Confers Type 1 Regulatory T Cell (Tr1) Phenotype and Function to Human CD4$^+$ T Cells

Grazia Andolfi[1], Georgia Fousteri[1], Maura Rossetti[1,2], Chiara F Magnani[1], Tatiana Jofra[3], Grazia Locafaro[1], Attilio Bondanza[4], Silvia Gregori[1] and Maria-Grazia Roncarolo[1,2]

[1]San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Milan, Italy; [2]Università Vita-Salute San Raffaele, Milan, Italy; [3]San Raffaele Diabetes Research Institute (HSR-DRI), Division of Immunology, Transplantation and Infectious Diseases, San Raffaele Scientific Institute, Milan, Italy; [4]Experimental Hematology Unit, PIBIC, Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Milano, Italy

Type 1 regulatory T (Tr1) cells are an inducible subset of CD4$^+$ Tr cells characterized by high levels of interleukin (IL)-10 production and regulatory properties. Several protocols to generate human Tr1 cells have been developed *in vitro*. However, the resulting population includes a significant fraction of contaminating non-Tr1 cells, representing a major bottleneck for clinical application of Tr1 cell therapy. We generated an homogeneous IL-10–producing Tr1 cell population by transducing human CD4$^+$ T cells with a bidirectional lentiviral vector (LV) encoding for human IL-10 and the marker gene, green fluorescent protein (GFP), which are independently coexpressed. The resulting GFP$^+$ LV-IL-10–transduced human CD4$^+$ T (CD4$^{LV\text{-}IL\text{-}10}$) cells expressed, upon T-cell receptor (TCR) activation, high levels of IL-10 and concomitant low levels of IL-4, and markers associated with IL-10. Moreover, CD4$^{LV\text{-}IL\text{-}10}$ T cells displayed typical Tr1 features: the anergic phenotype, the IL-10, and transforming growth factor (TGF)-β dependent suppression of allogeneic T-cell responses, and the ability to suppress in a cell-to-cell contact independent manner *in vitro.* CD4$^{LV\text{-}IL\text{-}10}$ T cells were able to control xeno graft-versus-host disease (GvHD), demonstrating their suppressive function *in vivo*. These results show that constitutive over-expression of IL-10 in human CD4$^+$ T cells leads to a stable cell population that recapitulates the phenotype and function of Tr1 cells.

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INTRODUCTION

Interleukin-10 (IL-10) is a pleiotropic cytokine characterized by a broad spectrum of anti-inflammatory activities. Produced by several cells including T, B, and natural killer (NK) cells, dendritic cells (DC), macrophages, mast cells, neutrophils, and eosinophils, when bound to its receptor, IL-10 activates the STAT3-mediated signaling which results in the inhibition of different target genes.[1] IL-10 acts primarily on antigen-presenting cells inhibiting the upregulation of major histocompatibility complex class II and costimulatory molecules and the release of proinflammatory cytokines and chemokines, overall limiting antigen-presenting cell function.[2,3] IL-10 can also directly inhibit T-cell function and cytokine production,[4,5] chemotaxis,[6] and proliferation.[7] IL-10 is involved in the induction and maintenance of peripheral tolerance; specifically, it is implicated in the generation and suppressive function of a subset of adaptive regulatory T cells, namely Tr1.[8,9]

Tr1 cells were initially described in severe combined immunodeficient patients that developed long-term tolerance to stem cell allografts[10,11] and they were subsequently associated with disease prevention and cure in a variety of murine and human pathological conditions.[9] For instance, Tr1 cells were detected in the peripheral blood of β-thalassemic patients who developed persistent mixed chimerism associated with tolerance after allogeneic hematopoietic stem cell transplantation.[12] Tr1 cells are induced upon chronic exposure to antigen in the presence of IL-10 and are characterized by a distinct cytokine production profile, namely IL-10^{++}TGF-β$^+$IFN-γ$^+$IL-5$^+$IL-4$^-$IL-2$^{low/neg}$.[8,13] Tr1 cells suppress effector T cells *via* IL-10- and TGF-β-dependent mechanisms[8] and kill myeloid antigen-presenting cell *via* granzyme (GZ)-B and perforin production.[14]

The interest in regulatory T cell-based therapy for treating several immune-mediated pathologies has recently increased. The use of freshly isolated natural CD25$^+$ Treg, expanded CD25$^+$ Treg, and Tr1 cells has been already tested in proof-of-concept clinical trials.[15–17] A number of protocols for *in vitro* induction of human Tr1 cells have been described, where cells are activated in the presence of either recombinant IL-10 and interferon (IFN)-α,[18] or a combination of immunosuppressive drugs (vitamin D3 and dexamethasone),[19] or anti-CD3 and anti-CD46 monoclonal antibodies,[20] or recombinant transforming growth factor (TGF)-β and IL-27.[21] Tr1 cells can also be differentiated by repetitive stimulation of naive T cells with allogeneic immature monocyte-derived DC[22] or

The first two authors and the last two authors contributed equally to this work.

Correspondence: *Silvia Gregori, San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Via Olgettina 58, Milan 20132, Italy. E-mail: silvia.gregori@hsr.it or Maria-Grazia Roncarolo, San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Via Olgettina 58, Milan 20132, Italy. E-mail: maria-grazia.roncarolo@hsr.it*

by a single stimulation with IL-10-treated tolerogenic DC, named DC-10.[23,24] In addition, virally activated plasmacytoid DC can induce Tr1 cells *in vitro*.[25] In most protocols, however, only a small fraction of the cells produces IL-10, suggesting that few Tr1 cells can be obtained. The presence of contaminating effector T cells in the resulting population represents a major barrier toward the clinical application of adoptive Tr1 cell therapy in humans. One potential strategy to overcome this limitation is to generate a homogeneous IL-10–producing T cell population through direct IL-10 gene transfer. Previous murine studies showed that after retroviral gene transfer of either murine or viral IL-10, $CD4^+$ T cells acquire the ability to suppress T cell-mediated responses *in vitro*[26] and *in vivo* in various T cell-mediated diseases, *i.e.,* rheumatoid arthritis,[27,28] colitis,[29] and allergen-induced Th2-dependent airway hyperreactivity.[30] Notably, in these studies, retroviral vectors were used to promote IL-10 expression in murine $CD4^+$ TCR transgenic cells and in one report in $CD4^+$ polyclonal cells, but no extensive characterization of the resulting murine IL-10–transduced T cells was performed. Lentiviral vectors (LVs) encoding for IL-10 were directly injected intragraft in preclinical experimental models of heart transplantation[31] and intracorneal to prevent uveitis.[32] In these models specific downregulation of the immune response without general immunosuppression was observed.

LVs have been shown to guarantee higher transduction efficiency in T cells with preservation of immune competence *in vitro*.[33,34] To generate a homogeneous population of Tr1 cells *in vitro*, which can be administered *in vivo*, we transduced human $CD4^+$ T cells with LV encoding human IL-10. To this aim, we developed a bidirectional LV, similar to the one previously described,[35] where expression of IL-10 and that of a marker gene, in this case green fluorescent protein (GFP), are driven by different promoters (LV-IL-10/GFP). We found that the resulting $CD4^{LV\text{-}IL\text{-}10}$ T cells produced high levels of IL-10, upregulated the expression of IL-10R and other Tr1-related tolerogenic markers (*i.e.,* human leukocyte antigen-G (HLA-G), inducible costimulator (ICOS), inducible costimulator-ligand (ICOS-L)), were anergic and acquired lytic and suppressive activities *in vitro* and *in vivo* in NOD.scid mice in which xeno graft-versus-host disease (xeno-GvHD) was induced. These results demonstrate that constitutive over-expression of hIL-10 confers Tr1 cell features to human $CD4^+$ T cells.

RESULTS

Bidirectional LVs encoding for hIL-10 and GFP can efficiently transduce human $CD4^+$ T cells, which upon expansion secrete high levels of IL-10

To generate LVs co-encoding for IL-10 and GFP, as marker gene, (LV-IL-10/GFP), we cloned the complementary DNA of human IL-10 into a bidirectional LV[35] under the control of the PGK promoter whereas the mCMV promoter controlled GFP expression, as shown in **Figure 1a**. A bidirectional LV co-encoding for GFP (LV-GFP) and ΔNGFR (in IL-10 position) was used as control (**Figure 1a**). To obtain a high transduction rate, human $CD4^+$ T cells were preactivated for 48 hours with low concentrations of soluble anti-CD3 (30 ng/ml) and anti-CD28 (1 µg/ml) monoclonal antibodies (mAbs) in the presence of rhIL-2 (50 U/ml). Preactivated cells were then infected with LVs overnight (multiplicity of infection: 20). Forty to eighty percent of $CD4^+$ T cells were transduced, as confirmed by GFP expression (**Figure 1b**). Due to the presence of the bidirectional promoter, hIL-10/ΔNGFR and GFP genes are translated into separate proteins, but their expression is coordinated and the levels of expression are comparable (**Figure 1c**), as previously demonstrated.[35] Quantitative real-time reverse transcription-PCR for IL-10 and GFP performed on $CD4^+$ T cells transduced with LV-IL-10/GFP ($CD4^{LV\text{-}IL\text{-}10}$) confirmed these findings (data not shown). $CD4^{LV\text{-}IL\text{-}10}$ T cells acquired the ability to release significantly higher levels of IL-10 compared to $CD4^+$ T cells transduced with LV-GFP ($CD4^{LV\text{-}GFP}$) (on average 18.1 ng/ml versus 0.1 ng/ml in culture supernatant 6 days after transduction, respectively, $n = 8$, $P = 0.0009$, data not shown).

LV-IL-10–transduced $CD4^+$ T cells express Tr1-related phenotypic markers

The LV-IL-10/GFP-transduced $CD4^+$ T ($CD4^{LV\text{-}IL\text{-}10}$) cells were fluorescence-activated cell sorted according to CD4 and GFP expression and expanded *in vitro* using feeder mixture, as described in Materials and Methods. In all experiments cells transduced with LV-GFP ($CD4^{LV\text{-}GFP}$) and untransduced cells were included as controls. Notably, the expansion of $CD4^{LV\text{-}GFP}$ T cells was significantly higher than that of $CD4^{LV\text{-}IL\text{-}10}$ T cells (fold increase of 10.4 ± 4.6 versus 3.6 ± 2.2, respectively, $n = 5$, $p = 0.0313$), suggesting that in the latter expansion was impaired by IL-10 secretion (data not shown). The vector copy number was on average 1.6 ± 0.3 for sorted $CD4^{LV\text{-}GFP}$ and 2.5 ± 0.3 for sorted $CD4^{LV\text{-}IL\text{-}10}$ T cells (data not shown). *In vitro* expanded $CD4^{LV\text{-}IL\text{-}10}$ and $CD4^{LV\text{-}GFP}$ T cells displayed a memory phenotype ($CD45RO^+$, $CD45RA^-$) but the $CD4^{LV\text{-}IL\text{-}10}$ T cells showed predominantly a central memory profile ($CD62L^{hi}$) (**Figure 2a** and **Supplementary Figure S1**). No significant differences in the expression of CD40L, CD40, CD25, FOXP3, CD161, and CD137 were observed between $CD4^{LV\text{-}IL\text{-}10}$ and $CD4^{LV\text{-}GFP}$ T cells (**Figure 2a** and **Supplementary Figure S2a**). Interestingly, expression of markers associated with IL-10 (**Figure 2b**) such as PD-1,[36] ICOS-L, ICOS,[37] and IL-10R, was significantly higher in $CD4^{LV\text{-}IL\text{-}10}$ compared to $CD4^{LV\text{-}GFP}$ T cells, although a variability among T-cell lines generated from different donors was observed, (on average $PD\text{-}1^+$ cells 16.2 ± 3.8 versus 7.1 ± 1.6%, $n = 6$, $P = 0.053$, $ICOS\text{-}L^+$ cells 11.9 ± 2.4 versus 4.5 ± 0.66%, $n = 14$, $P = 0.0074$, $ICOS^+$ cells 6.6 ± 1.6 versus 2.9 ± 0.82%, $n = 14$, $P = 0.0047$, and $IL\text{-}10R^+$ cells 8.6 ± 1.5 versus 3.5 ± 0.75%, $n = 11$, $P = 0.0058$, **Figure 2b**). The relative low expression of IL-10R on $CD4^{LV\text{-}IL\text{-}10}$ T cells might be dependent on the binding of IL-10, which increases the receptor's occupancy limiting the labeling by anti-IL-10R antibody. To overcome this limitation, we measured the IL-10R expression levels by real-time reverse transcription-PCR (see **Supplementary Materials and Methods**). Results confirmed that $CD4^{LV\text{-}IL\text{-}10}$ T cells express significantly higher levels of IL-10R messenger RNA as compared to control $CD4^{LV\text{-}GFP}$ T cells (on average a fold increase expression of 38 ± 14 versus 11.9 ± 6.4, $n = 3$, in $CD4^{LV\text{-}IL\text{-}10}$ and $CD4^{LV\text{-}GFP}$ T cells, respectively, **Supplementary Figure S3**). The expression of the non-classical HLA class I molecule HLA-G on $CD4^{LV\text{-}IL\text{-}10}$ T cells was also investigated, because we recently demonstrated that IL-10 derived from tolerogenic DC promotes the upregulation of HLA-G on T cells.[24] $CD4^{LV\text{-}IL\text{-}10}$ T cells expressed significantly higher levels of HLA-G than $CD4^{LV\text{-}GFP}$ T cells (on average 26.6 ± 4.8 versus 8.7 ± 2.4%, $n = 7$, $P = 0.0063$, **Figure 2b**). The effect of IL-10 was specific for HLA-G, because the expression of

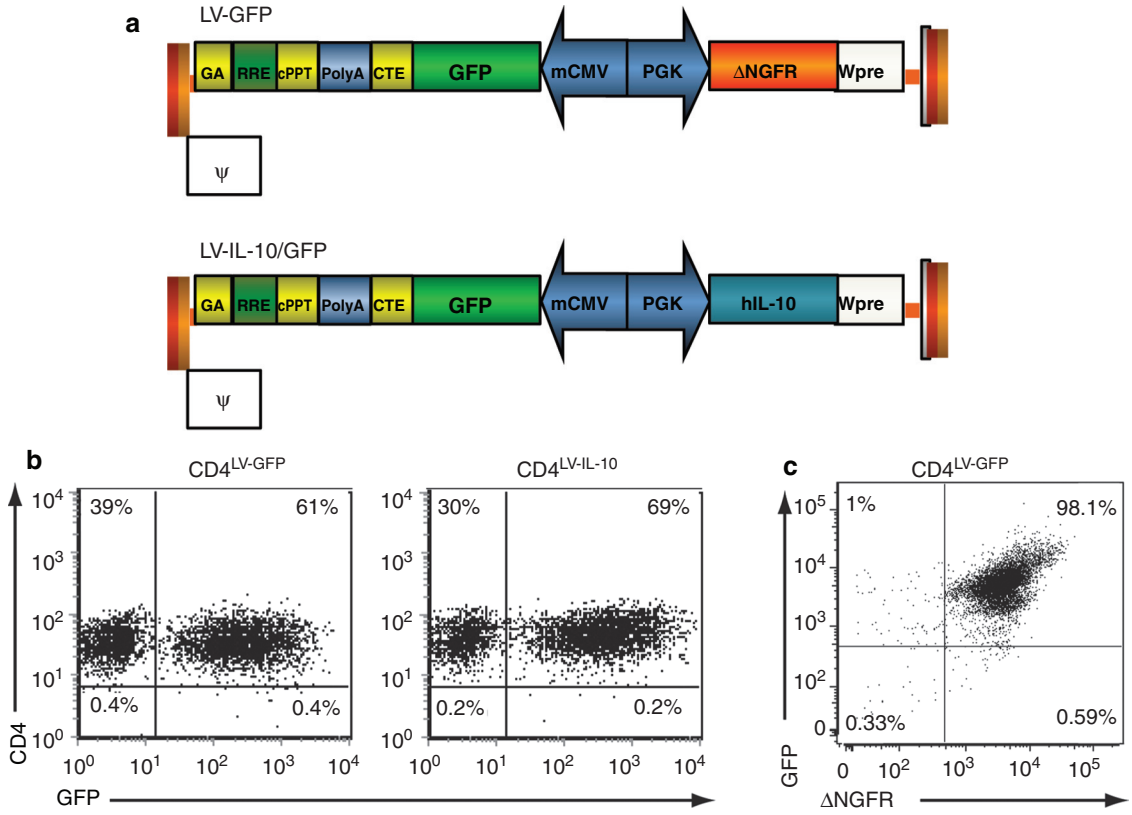


Figure 1 Bidirectional LV-IL-10/GFP and LV-GFP efficiently transduce human CD4+ T cells. (**a**) Scheme of the lentiviral vectors. hIL-10 cDNA replaced the ΔNGFR coding sequence in the LV-GFP vector to obtain LV-IL-10/GFP. The presence of the bidirectional promoter (mCMV/PGK) allows the coregulated expression GFP and IL-10/ΔNGFR genes. Ψ, encapsidation signal including the 5′ portion of GAG gene (GA); cPPT, central poly-purine tract; CTE, constitutive transport element; polyA, poly-adenylation site from the simian Virus 40; RRE, Rev-responsive element; WPRE, woodchuck hepatitis virus post-transcription regulatory element. (**b**) Human CD4+ or CD4+CD45RO− T cells were activated with soluble anti-CD3, soluble anti-CD28 mAbs, and rhIL-2 for 48 hours before transduction with LV-GFP or LV-IL-10/GFP at MOI of 20. After 6 days, transduced cells were analyzed by FACS. The frequency of GFP+ cells are indicated on top of each quadrant. One representative donor of at least eight analyzed is presented. (**c**) Coordinated expression of ΔNGFR/IL-10 and GFP. FACS-sorted CD4+GFP+ cells were expanded in feeder mixture as described in Materials and Methods. At the end of the feeder mixture, cells were analyzed for the expression of the ΔNGFR and GFP by flow cytometry. cDNA, complementary DNA; CMV, cytomegalovirus; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; IL, interleukin; LV, lentiviral vector; mAb, monoclonal antibody; MOI, multiplicity of infection.

classical HLA class I molecules was comparable between $CD4^{LV\text{-}GFP}$ and $CD4^{LV\text{-}IL\text{-}10}$ T cells (Figure 2a).

Upregulation of above IL-10–related genes and in particular of HLA-G increased after long-term *in vitro* culture (more than three rounds of feeders), suggesting that the Tr1 phenotype was maintained by the autocrine action of IL-10. Indeed, IL-10 levels were significantly elevated in $CD4^{LV\text{-}IL\text{-}10}$ long-term cultures ranging from 5 to 40 ng/ml (data not shown). Altogether, our results show that stable enforced expression of IL-10 in human CD4+ T cells by means of LV transduction induces a Tr1 cell phenotype, with high expression of PD-1, ICOS-L, ICOS, IL-10R, and HLA-G.

LV-IL-10–transduced CD4+ T cells display a Tr1 cytokine production profile

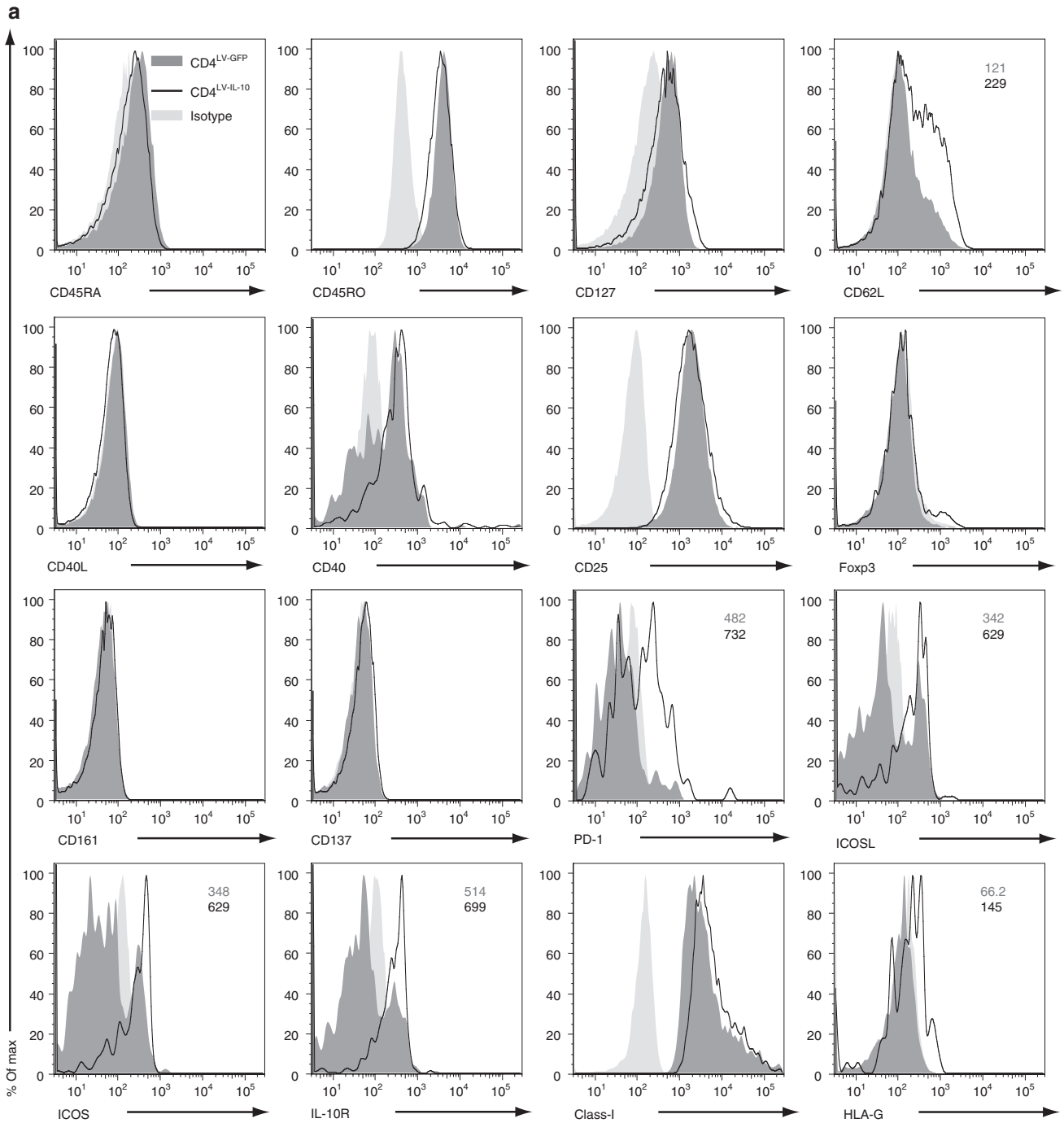
We next determined the cytokine production profile of GFP+ sorted and *in vitro* expanded $CD4^{LV\text{-}IL\text{-}10}$ in comparison to $CD4^{LV\text{-}GFP}$ T cells unstimulated or upon polyclonal stimulation with anti-CD3/anti-CD28 mAbs in the presence or absence of rhIL-2 or after stimulation with LAK (leukocyte activation cocktail: TPAphorbol 12-myristate 13-acetate (PMA)/ionomycin). As shown in Figure 3a, upon activation, LV-IL-10–transduced cells but not controls ($CD4^{LV\text{-}GFP}$) concomitantly upregulated IL-10 and GFP expression. The increased production of IL-10 was more evident when the mean fluorescence intensity (MFI) for IL-10-PE was plotted (Figure 3b,c). Results obtained with intracellular staining were further confirmed by measurement of IL-10 in culture supernatants. $CD4^{LV\text{-}IL\text{-}10}$ T cells secrete spontaneously significantly higher levels of IL-10 as compared to $CD4^{LV\text{-}GFP}$ T cells (on average 0.51 ± 0.44 versus 0.007 ± 0.009 ng/ml respectively, $n = 10$, $P = 0.0012$), which increased upon TCR stimulation (on average 4.7 ± 1.5 versus 1.3 ± 0.42 ng/ml, $n = 13$, $P = 0.04$, (Figure 3d). These results confirm the Tr1 "nature" of $CD4^{LV\text{-}IL\text{-}10}$ T cells, which were able to produce high amounts of IL-10 in an inducible manner, upon activation.

We further characterized the cytokine signature of $CD4^{LV\text{-}IL\text{-}10}$ T cells. In addition to secrete significantly higher amounts of IL-10 compared to $CD4^{LV\text{-}GFP}$, $CD4^{LV\text{-}IL\text{-}10}$ T cells secrete significantly higher levels of TGF-β1 (on average 1.05 ± 0.45 versus 0.29±0.22 ng/ml, $n = 2$, $P = 0.042$) and significantly lower amounts of IL-4 (on average 70 ± 34 versus 275 ± 58 pg/ml, $n = 12$, $P = 0.0063$) and IL-5 (on average 8.2 ± 2.6 versus 18.9 ± 2.4 ng/ml, $n = 9$, $P = 0.0131$; Figure 4). No statistically significant differences in IL-2, IFN-γ, and tumor necrosis factor-α production were observed

(Figure 4). In line with their cytokine production profile, $CD4^{LV\text{-}IL\text{-}10}$ T cells expressed similar levels of T-bet, but lower levels of Gata-3, compared to $CD4^{LV\text{-}GFP}$ T cells ($n = 3$, **Supplementary Figure S4**). These results indicate that human $CD4^+$ T cells stably transduced with IL-10 acquire a cytokine production profile consistent with that of Tr1 cells.

LV-IL-10–transduced $CD4^+$ T cells are anergic, suppress allogeneic responses *in vitro* and are cytotoxic

A prominent characteristic of Tr1 cells is that they are anergic upon re-stimulation *in vitro*. The proliferative capacity measured by thymidine incorporation of $CD4^{LV\text{-}IL\text{-}10}$ T cells upon anti-CD3/CD28 stimulation *in vitro* was lower compared to that of $CD4^{LV\text{-}GFP}$ T cells (6 ± 2.3 versus $26.1 \pm 11.7 \times 10^3$ cpm, $n = 6$), with a mean reduction in proliferation of $67.1 \pm 23.7\%$ ($P < 0.005$) (Figure 5a). The anergic state of $CD4^{LV\text{-}IL\text{-}10}$ T cells was reverted by addition of 100 U/ml of rhIL-2 to the culture (Figure 5b). Next, we evaluated the anergic state of $CD4^{LV\text{-}IL\text{-}10}$ T cells by measuring their proliferation in the presence of third party allogeneic peripheral blood mononuclear cells (PBMCs). $CD4^{LV\text{-}IL\text{-}10}$ T cells were stained with Vybrant or eFluor670 and stimulated with anti-CD3/CD28 mAbs in the absence or presence of unlabeled allogeneic PBMCs at increasing ratio and their proliferation was determined by flow



cytometry. Results in **Figure 5c** show that $CD4^{LV\text{-}IL\text{-}10}$ T cells are profoundly anergic also in the presence of allogeneic PBMCs. $CD4^{LV\text{-}GFP}$ T cells under the same culture conditions showed increased proliferation, especially at low PBMCs number.

The suppressive activity of LV-IL-10–transduced cells *in vitro* was next examined. Allogeneic Vybrant/eFluor670-stained $CD4^+$ responder T cells were stimulated with anti-CD3/CD28 mAbs alone or in combination with $CD4^{LV\text{-}IL\text{-}10}$ or control $CD4^{LV\text{-}GFP}$ T cells at 1:1 ratio and their proliferation (Vybrant/eFluor670 dilution) was determined by flow cytometry. Results in **Figure 5d,e** show that proliferation of $CD4^+$ T cells was suppressed by the addition of allogeneic $CD4^{LV\text{-}IL\text{-}10}$ T cells (one representative donor shown in **Figure 5d**; mean suppression of proliferation of $CD4^+$ responder cells: 39.25 ± 5.17, $n = 22$, $P < 0.0001$, **Figure 5e**). $CD4^{LV\text{-}IL\text{-}10}$ T cells suppress proliferation of responder $CD4^+$ T cells independently from their proliferative capacity. Moreover, the proliferation of purified allogeneic eFluor670-stained $CD8^+$ responder cells stimulated with anti-CD3/CD28 mAbs was also significantly suppressed by the addition of $CD4^{LV\text{-}IL\text{-}10}$ T cells (one representative donor shown in **Figure 5f**; mean suppression of proliferation of $CD8^+$ responder cells: 47.22 ± 13.67%, $n = 4$, $P = 0.0136$, **Figure 5g**). Addition of anti-IL-10R in combination with anti-TGF-β1/2/3 neutralizing antibodies partially reversed the suppression of $CD4^+$ responder cells (**Supplementary Figure S5**) and $CD8^+$ responder cells (data not shown) mediated by $CD4^{LV\text{-}IL\text{-}10}$ T cells, while transwell experiments demonstrated that suppression mediated by $CD4^{LV\text{-}IL\text{-}10}$ T cells is maintained also in the absence of cell-to-cell contact (**Supplementary Figure S5**).

We then investigated GZ-A and GZ-B expression levels along with the cytotoxic activity by $CD4^{LV\text{-}IL\text{-}10}$ T cells and $CD4^{LV\text{-}GFP}$ T cells as control. As shown in **Figure 6a,c**, $CD4^{LV\text{-}IL\text{-}10}$ T cells expressed similar levels of GZ-A but significantly higher levels of GZ-B compared to $CD4^{LV\text{-}GFP}$ T cells (on average 4.843 ± 647 versus 1.366 ± 435 mean fluorescence intensity, $n = 6$, $P = 0.0012$). $CD4^{LV\text{-}IL\text{-}10}$ lysed U937 target cells, a monocytic cell line, but not K562 cells, an erythroleukemic cell line, similarly to what recently observed with polarized Tr1 cell lines (**Figure 6d,e**, and ref. 14). Notably, lytic activity of $CD4^{LV\text{-}IL\text{-}10}$ T cells against U937 target cells was significantly higher compared to that elicited by $CD4^{LV\text{-}GFP}$ T cells at 100:1, 33:1, 11:1, and 4:1 E:T ratio. We next evaluated the degranulation of $CD4^{LV\text{-}IL\text{-}10}$ and $CD4^{LV\text{-}GFP}$ T cells by the coexpression of GZ-B and the lysosomal-associated membrane protein 1 (LAMP-1 or CD107a), a marker of cytotoxic degranulation in natural killer cells and cytotoxic T lymphocytes.[14] When $CD4^{LV\text{-}IL\text{-}10}$ T cells were cocultured with U937 target cells, a significant proportion of cells coexpressed CD107a and GZ-B (on average 16.7%, $n = 2$) consistent with their lytic activity (**Figure 6f**). In contrast, $CD4^{LV\text{-}GFP}$ T cells displayed low coexpression of CD107a/GZ-B when cocultured with U937 target cells (on average <0.1%), despite degranulating (on average 15.7% of $CD107^+GZ\text{-}B^-$ cells, $n = 2$). As expected, percentages of $CD107a^+GZ\text{-}B^+$ cells within the $CD4^{LV\text{-}IL\text{-}10}$ T cells cocultured with K562 cells were low, confirming their ability to lyse specifically cells of myeloid origin. In conclusion, stable over-expression of IL-10 in human $CD4^+$ T cells promotes the generation of a homogeneous cell population that recapitulates the function of Tr1 cells.

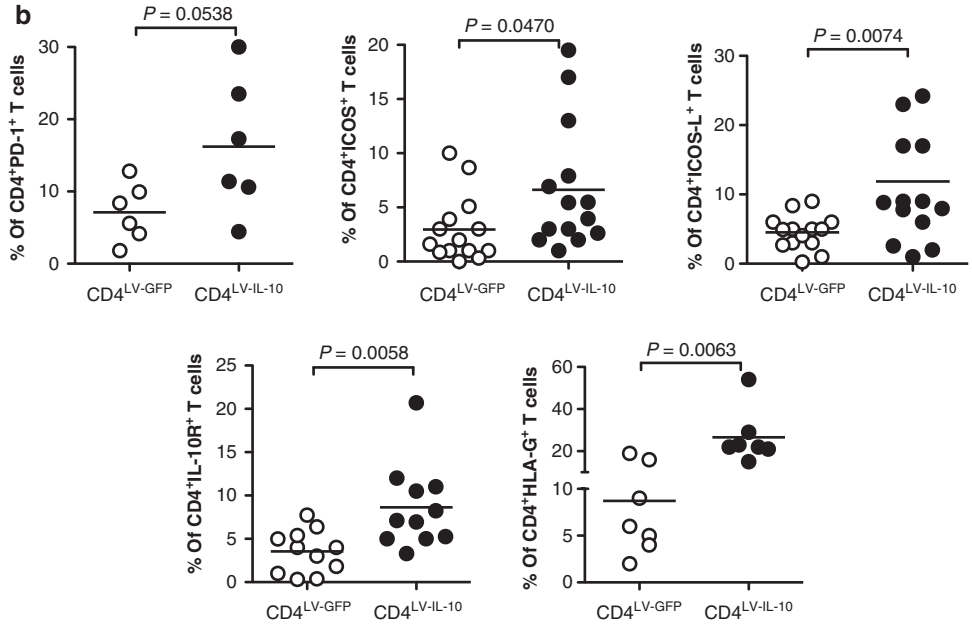


Figure 2 LV-IL-10–transduced $CD4^+$ T cells express Tr1-related markers. FACS-sorted $CD4^+GFP^+$ cells were expanded in feeder mixture for up to three rounds as described in Materials and Methods. (**a**) Unstimulated $CD4^{LV\text{-}GFP}$ (solid gray histogram) and $CD4^{LV\text{-}IL\text{-}10}$ (black line-overlay) T cells were analyzed for the expression of the indicated markers by flow cytometry after gating on the $CD4^+/GFP^+$ population (solid light gray histogram, isotype control). Numbers in upper corner of some plots indicate the MFI on $CD4^+GFP^+$ gated cells in $CD4^{LV\text{-}GFP}$ (gray fonts) and $CD4^{LV\text{-}IL\text{-}10}$ (black fonts) T cells. (**b**) Collective results from individual donors are presented. Black lines represent the mean percentages of $CD4^+PD\text{-}1^+$, $CD4^+ICOS^+$, $CD4^+ICOS\text{-}L^+$, $CD4^+IL\text{-}10R^+$, and $CD4^+HLA\text{-}G^+$ in $CD4^{LV\text{-}GFP}$ and $CD4^{LV\text{-}IL\text{-}10}$ T cells. FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; HLA-G, human leukocyte antigen-G; ICOS-L, inducible costimulator-ligand; IL, interleukin; LV, lentiviral vector; MFI, mean fluorescence intensity.

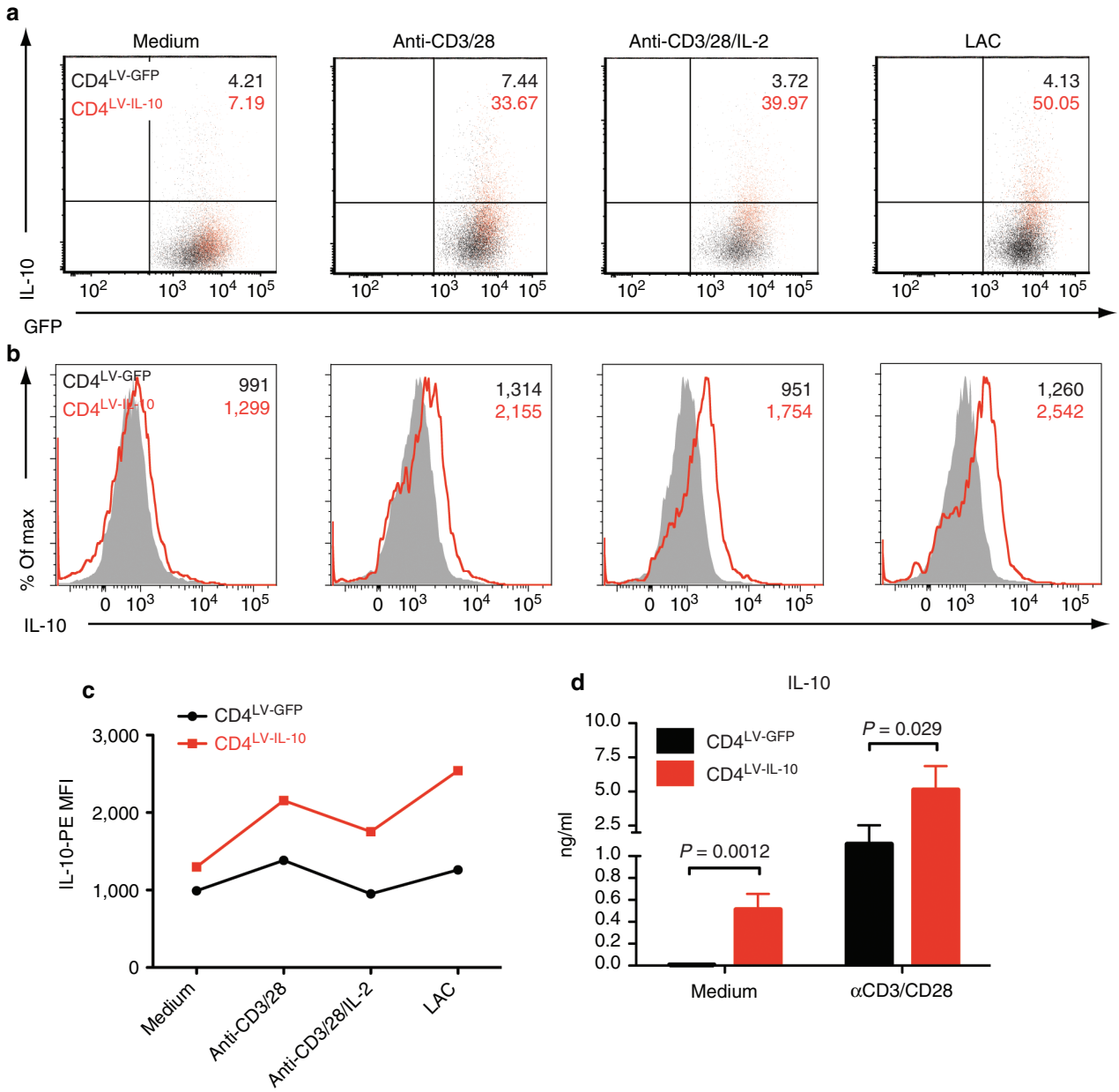


Figure 3 LV-IL-10–transduced CD4+ T cells produce IL-10 upon activation. FACS-sorted $CD4^+GFP^+$ were expanded with feeder mixture for up to three rounds. After resting, cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 mAbs in the presence or absence of rhIL-2 or with LAC (leukocyte activation cocktail: PMA/IONO) for 4 hours before intracellular staining for IL-10. (**a**) Dot plot overlay depicting IL-10–producing cells after gating on $CD4^{LV\text{-}GFP}$ (black dots) and $CD4^{LV\text{-}IL\text{-}10}$ (red dots) T cells. The percentage of $IL\text{-}10^+$ cells is depicted on the upper right corner of the panels for $CD4^{LV\text{-}GFP}$ (black fonts) and $CD4^{LV\text{-}IL\text{-}10}$ (red fonts). (**b**) Histogram overlay showing the expression levels of IL-10 on $CD4^+GFP^+$ gated cells in $CD4^{LV\text{-}GFP}$ (black line) or $CD4^{LV\text{-}IL\text{-}10}$ (red line) cell lines. The IL-10 mean fluorescence intensities (MFI) are depicted in the upper right corner. (**c**) The IL-10-PE MFI in $CD4^{LV\text{-}GFP}$ (black line) and $CD4^{LV\text{-}IL\text{-}10}$ (red line) T cells activated as in **a**. (**d**) $CD4^{LV\text{-}IL\text{-}10}$ T cells produce constitutive levels of IL-10, which are increased upon activation. $CD4^{LV\text{-}GFP}$ and $CD4^{LV\text{-}IL\text{-}10}$ T cells were left inactivated or stimulated with immobilized anti-CD3 and soluble anti-CD28 mAbs. After 48 hours IL-10 levels in culture supernatant were measured by ELISA. All samples were tested in duplicate-triplicate. Mean ± SD of $n = 10$ donors analyzed are presented. ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; IL, interleukin; IONO, ionomycin; LV, lentiviral vector; mAb, monoclonal antibody, PMA, phorbol 12-myristate 13-acetate.

LV-IL-10–transduced CD4+ T cells control xeno GvHD

The *in vivo* immunomodulatory properties of LV-IL-10–transduced $CD4^+$ T cells were investigated in a humanized model of xeno-GvHD. The suppressive activity of $CD4^{LV\text{-}IL\text{-}10}$ T cells was tested after adoptive transfer in previously conditioned NOD.scid mice in which lethal xeno-GvHD was induced by transfer of human peripheral blood $CD4^+$ T cells. In NOD.scid mice conditioned with nonlethal irradiation followed by an anti-natural killer depleting antibody infusion,[38] $CD4^{LV\text{-}IL\text{-}10}$ T cells and control $CD4^{LV\text{-}GFP}$ T cells were injected at two different concentrations (1×10^6 and 5×10^6 cells) concomitantly with the GvHD-inducing allogeneic $CD4^+$ T cells (5×10^6 cells). As shown in **Figure 7a,b**, all control animals (effector $CD4^+$ cells alone or effector $CD4^+$ cells infused together with $CD4^{LV\text{-}GFP}$

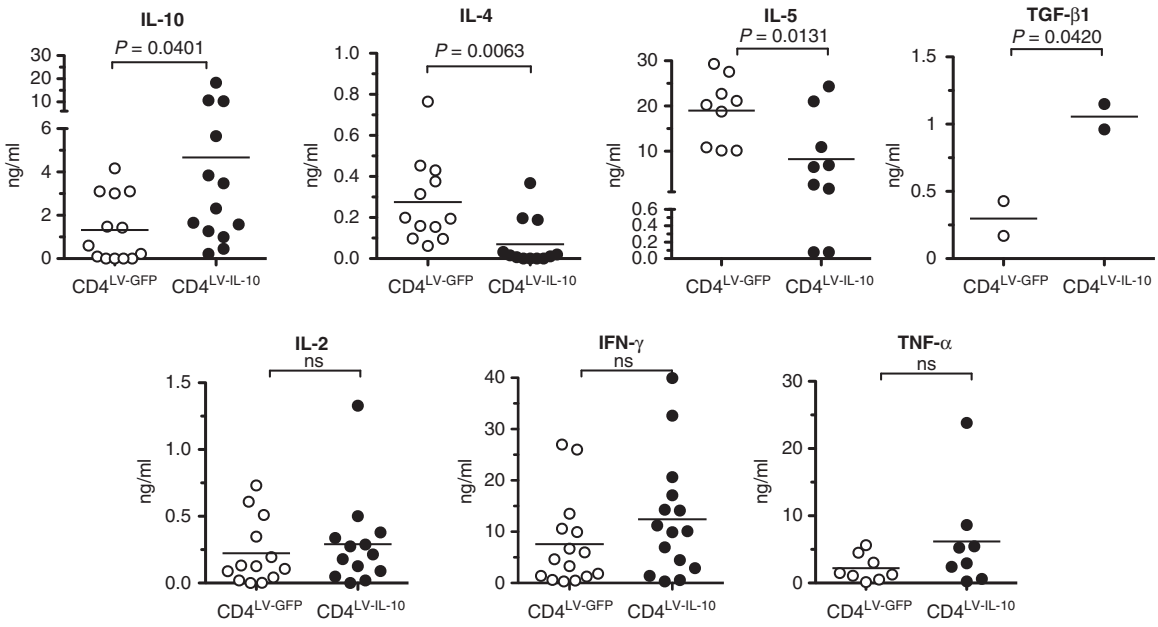


Figure 4 LV-IL-10–transduced CD4+ T cells display a Tr1 cytokine production profile. FACS-sorted and feeder expanded CD4+GFP+ cells were stimulated with immobilized anti-CD3 and soluble anti-CD28 mAbs. Cultures supernatants were collected and levels of IL-2 (24 hours), IL-4, IL-5, TGF-β1, IL-10, IFN-γ, and TNF-α (48 hours) were determined by ELISA or Bioplex. All samples were tested in duplicate-triplicate. Results from each of the donors analyzed are presented. Black lines represent the mean percentages of cytokine production. Statistical significant values are indicated, ns = not statistically significant. ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; IFN, interferon; IL, interleukin; LV, lentiviral vector; mAb, monoclonal antibody; TGF, transforming growth factor; TNF, tumor necrosis factor.

T cells) developed lethal xeno-GvHD, while the majority (six out of eight) of NOD.scid mice that received human CD4+ cells together with 5 × 10⁶ $CD4^{LV\text{-}IL\text{-}10}$ T cells did not (**Figure 7a**). Notably, coinjection of lower amounts of $CD4^{LV\text{-}IL\text{-}10}$ T cells (1 × 10⁶) with effector CD4+ cells was not sufficient to control xeno-GvHD (**Figure 7b**).

Xeno-GvHD development was also evaluated by weight loss and human chimerism. Results showed that the body weight of two NOD.scid mice coinjected with human CD4+ cells and high number of $CD4^{LV\text{-}IL\text{-}10}$ T cells (5 × 10⁶) was better maintained, while in the remaining mice of the same group a delay in the body weight loss was observed (**Figure 7c**). As expected, coinjection of 5 × 10⁶ $CD4^{LV\text{-}GFP}$ T cells with GvHD-inducing allogeneic CD4+ T cells (5 × 10⁶ cells) did not inhibit the weight loss of the recipient mice (**Figure 7d**). Human chimerism correlated with xeno-GvHD symptoms, as it is shown in **Figure 7e**. In mice that were protected or delayed from GvHD by $CD4^{LV\text{-}IL\text{-}10}$ T cells, lower levels of human chimerism were observed compared to control mice injected with human CD4+ T cells alone or in combination with $CD4^{LV\text{-}GFP}$ T cells. These findings show that $CD4^{LV\text{-}IL\text{-}10}$ T cells are capable to control xeno-GvHD by inhibiting the expansion of human CD4+ T cells. Interestingly, tracking $CD4^{LV\text{-}IL\text{-}10}$ (GFP^+) cells *in vivo* in the blood of the infused mice showed that these cells did not expand but rather decline with time (data not shown), suggesting that $CD4^{LV\text{-}IL\text{-}10}$ T cells acted promptly upon initial encounter with the GvHD-inducing CD4+ T cells. Taken together, these results indicate that stable over-expression of IL-10 confers not only Tr1 features to human CD4+ T cells but also potent *in vivo* regulatory properties to control human CD4-induced lethal xeno-GvHD.

DISCUSSION

In this report, we demonstrate that transduction with bidirectional LV encoding for hIL-10 and GFP (LV-IL-10/GFP), the latter as a marker for selection, confers to human CD4+ T cells a phenotype, cytokine production profile and a suppressive activity superimposable to that of Tr1 cells. The resulting $CD4^{LV\text{-}IL\text{-}10}$ T cells produce significantly higher levels of IL-10 and TGF-β1 but lower levels of IL-4 and IL-5 compared to $CD4^{LV\text{-}GFP}$ T cells, are anergic upon polyclonal stimulation, and suppress allogeneic T-cell proliferation *in vivo* and *in vitro*. Similarly to Tr1 cells,[14] $CD4^{LV\text{-}IL\text{-}10}$ T cells express high levels of GZ-B and lyse cells of myeloid origin.

Protocols currently established for the *in vitro* differentiation of Tr1 cells, including the use of recombinant hIL-10– or IL-10–derived from tolerogenic DC-10,[18,23,24] allow the generation of a cell population, which contains 10–15% of IL-10–producing Tr1 cells but also significant proportion of contaminating non-Tr1 cells. Presently, the absence of Tr1-specific surface markers hinders the possibility to purify the IL-10–producing Tr1 cells from this cell culture. The genetic modification of human CD4+ T cells with LV-IL-10/GFP represents therefore an attractive alternative approach to overcome this limitation and to generate a stable and more homogeneous population of human IL-10–producing regulatory T cells.

Preclinical studies previously demonstrated that over-expression of either murine or viral IL-10 into mouse TCR transgenic or polyclonal T cells after retroviral transduction confers the ability to suppress T cell-mediated responses *in vitro*[26] and upon adoptive transfer *in vivo* in murine models of autoimmunity and GvHD.[27,29,30,39] However, no biological characterization of

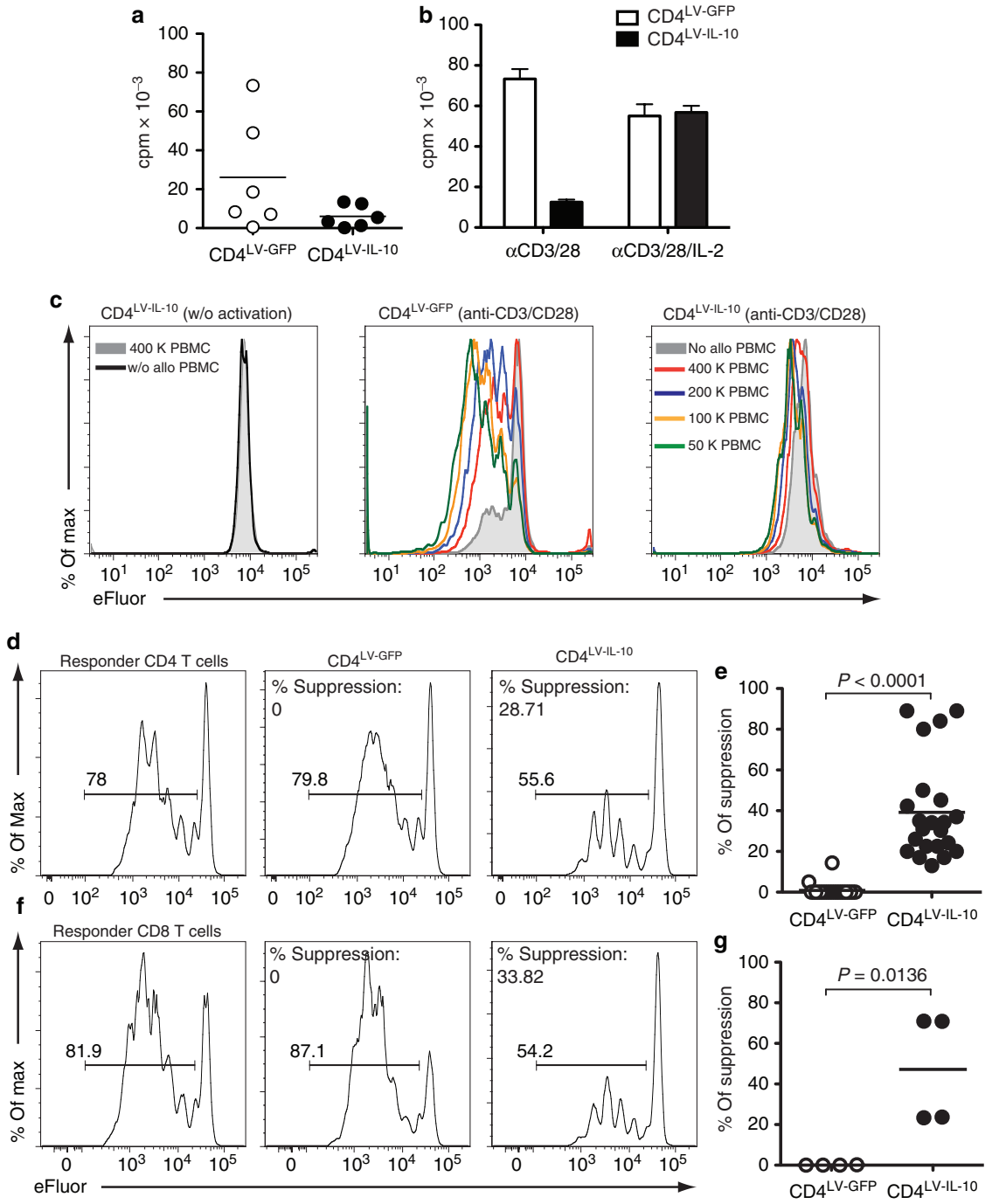


Figure 5 LV-IL-10–transduced CD4+ T cells are anergic and suppress the proliferation of allogeneic CD4+ and CD8+ responder cells. (**a**–**c**) $CD4^{LV\text{-}IL\text{-}10}$ T cell are anergic. FACS-sorted and feeder expanded $CD4^+GFP^+$ cells were stimulated with immobilized anti-CD3 and soluble anti-CD28 mAbs in the absence or presence of rhIL-2 (100 U/ml). [^{3}H]-thymidine was added after 3 days of culture for additional 16 hours. (**a**) Collective results from individual donors tested are shown, where black lines represent the mean thymidine incorporation (cpm × 10^{-3}). (**b**) One representative experiment of anergy reversal (from one donor) is shown. (**c**) $CD4^{LV\text{-}IL\text{-}10}$ and control $CD4^{LV\text{-}GFP}$ T cells were stained with Vybrant/eFluor670 and stimulated with anti-CD3/CD28 mAbs in the presence or absence of unlabeled allogeneic PBMCs at different ratios ($K = 10^3$), and their proliferation was determined by flow cytometry. (**d**–**g**) $CD4^{LV\text{-}IL\text{-}10}$ T cells suppress $CD4^+$ and $CD8^+$ T cell responses. FACS-sorted and feeder expanded $CD4^+GFP^+$ cells were tested for their efficacy to suppress the proliferation of allogeneic responders; $CD4^+$ or $CD8^+$ responder cells. Allogeneic $CD4^+$ responder T cells were labeled with Vybrant/eFluor670 and stimulated with immobilized anti-CD3 and anti-CD28 mAbs in the presence of $CD4^{LV\text{-}GFP}$ or $CD4^{LV\text{-}IL\text{-}10}$ T cell lines at 1:1 ratio. After 5 days of culture, the percentage of proliferating responder cells was determined by Vybrant/eFluor670 dilution with flow cytometry. (**d**) Results from one representative donor tested against $CD4^+$ responder cells are shown. (**e**) Cumulative data from all LV-transduced donors tested in suppression assay against $CD4^+$responder cells. (**f**) Results from one representative donor tested against $CD8^+$ responder cells are shown. (**g**) Cumulative data from all LV-transduced donors tested in suppression assay against $CD8^+$ responder cells. Black lines represent the mean percentages of suppression. The suppression mediated by $CD4^{LV\text{-}IL\text{-}10}$ T cells was calculated as follows: ((proliferation responder – proliferation transduced)/proliferation responder) × 100). Statistical significant values are indicated. cpm, counts per minute; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; IL, interleukin; LV, lentiviral vector; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell.

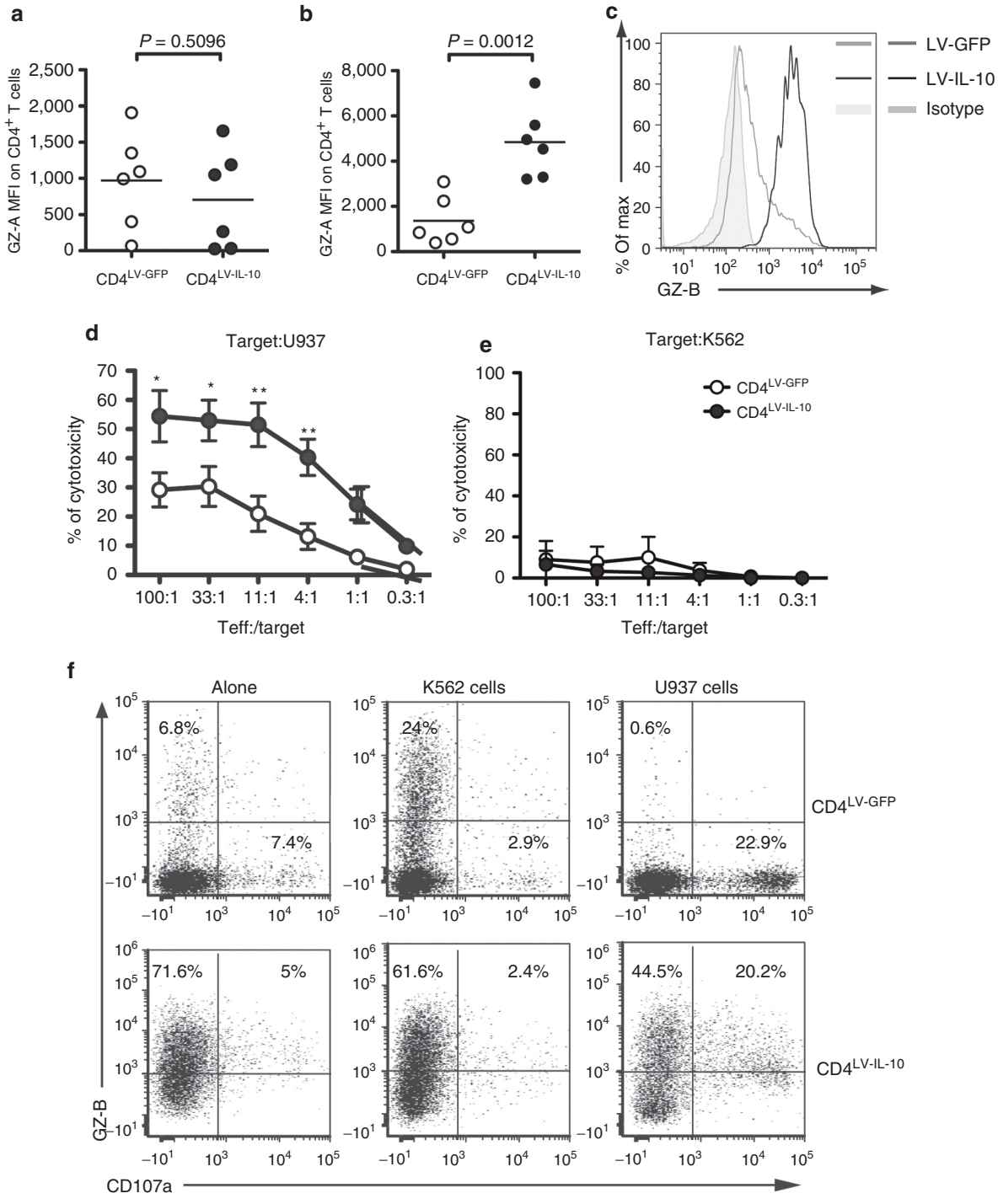


Figure 6 LV-IL-10–transduced CD4+ T cells express high levels of GZ-B and are cytotoxic. FACS-sorted and feeder expanded CD4+GFP+ cells were analyzed for the expression of (**a**) GZ-A and (**b**) GZ-B. Results from individual donors were analyzed and the mean result (black line) is shown. (**c**) Representative flow cytometry histogram overlay depicting the GZ-B expression levels in $CD4^{LV\text{-}GFP}$ and $CD4^{LV\text{-}IL\text{-}10}$ cells (gray solid histogram, isotype control). (**d**,**e**) The cytotoxic activity of $CD4^{LV\text{-}GFP}$ and $CD4^{LV\text{-}IL\text{-}10}$ T cells against U937 and K562 target cell lines was determined by ^{51}Cr-release assay. Average ± SE of five independent donors for U937 performed in triplicates is reported. *$P \leq 0.01$; **$P \leq 0.001$. (**f**) T cell degranulation was measured by coexpression of CD107a and GZ-B in $CD4^{LV\text{-}IL\text{-}10}$ and $CD4^{LV\text{-}GFP}$ T cells. One representative donor for U937 and for K562 is shown. FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; GZ, granzyme; IL, interleukin; LV, lentiviral vector; MFI, mean fluorescence intensity.

the resulting murine IL-10–transduced T cells was performed. Compared to retroviral vectors, LVs have higher transduction efficiency even in the absence of strong T-cell activation,[40] and lower oncogenic potential,[41] which renders them more suitable for the generation of modified T cells for clinical use. In this report, human CD4+ T cells were engineered *in vitro* with LVs to over-express hIL-10. The presence of the bidirectional promoter in the LV constructs results in coexpression of two separate

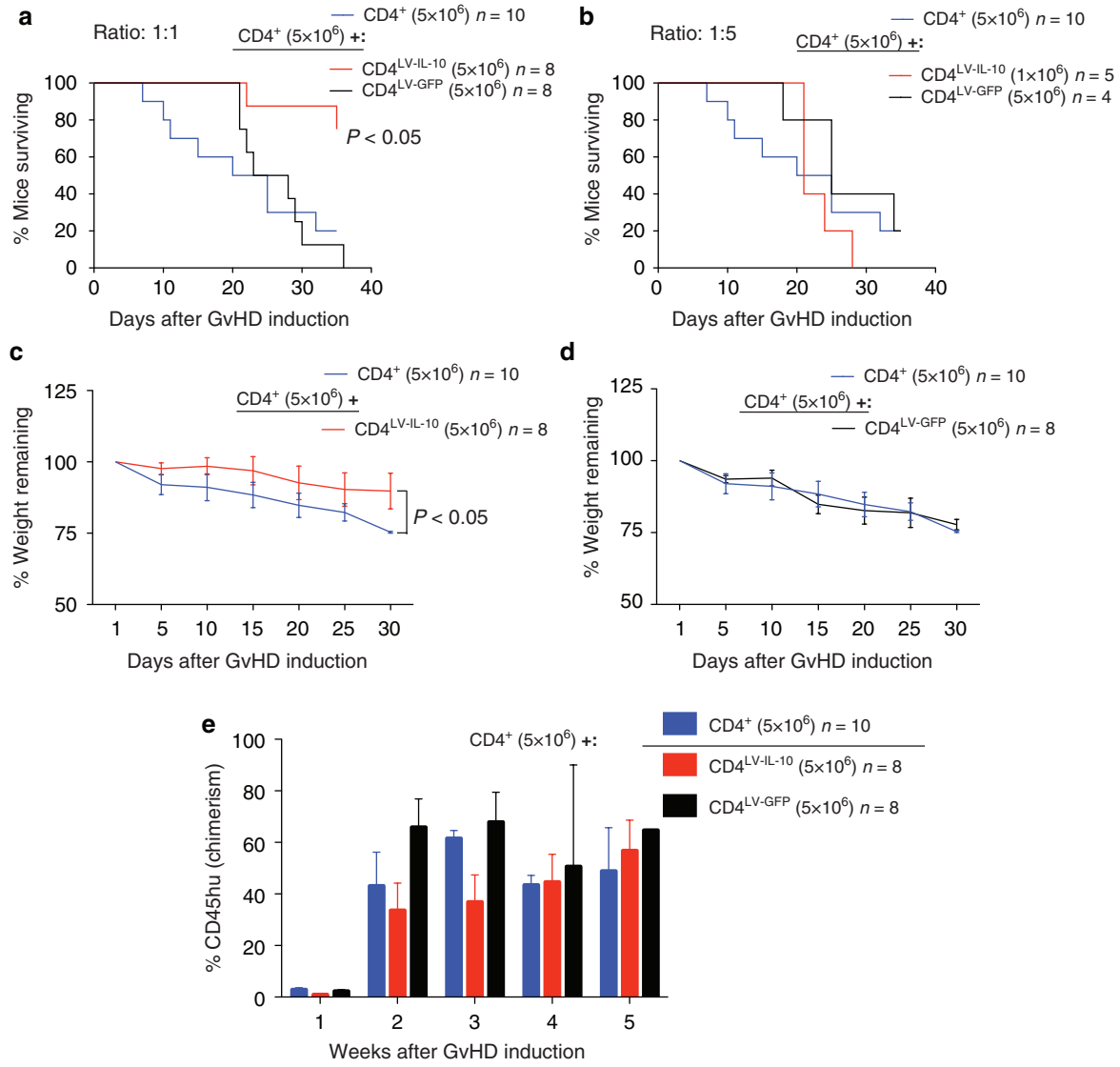


Figure 7 LV-IL-10–transduced CD4+ T cells reduce xeno graft-versus-host disease severity in a cell number-dependent manner. Eight- to ten-weeks-old NOD.scid female mice were irradiated and conditioned with the anti-NK antibody TMb-1 (anti-CD122, 1 mg/mouse). Subsequently, mice were infused intraperitoneally with (**a**,**c**,**d**) 5 × 10⁶ freshly isolated CD4+ T cells together with 5 × 10⁶ or (**b**) 1 × 10⁶ $CD4^{LV\text{-}GFP}$ or $CD4^{LV\text{-}IL\text{-}10}$ T cells. (**a**,**b**) Mice survival was followed over time after cell injection. (**c**,**d**) Weight loss was monitored at frequent intervals after cell injection. The percentage of weight change was determined as follows: 100 – ((initial weight – the weight measured at the indicated time points)/(initial weight) × 100). (**e**) The frequency of human CD45+ cells (hu chimerism) over the total lymphocytes in the blood of injected mice was measured weekly. GFP, green fluorescent protein; GvHD, graft-versus-host disease; IL, interleukin; LV, lentiviral vector; NK, natural killer; NOD, nonobese diabetic; scid, severe combined immunodeficiency.

transgenes[35]: IL-10 and a marker gene (GFP) that allows purification and tracking of transduced cells *in vivo*.

Human $CD4^{LV\text{-}IL\text{-}10}$ T cells acquire the typical Tr1 cytokine production profile: IL-10^{+++}, TGF-β^{+}, IL-5^{+}, IFN-γ^{+}, and IL-$4^{low/neg}$. In our system, the constitutive PGK promoter drives the constitutive low IL-10 expression, which is also dependent on T-cell activation. Indeed, $CD4^{LV\text{-}IL\text{-}10}$ T cells reach maximum levels of IL-10 production only after T-cell activation, induced by TCR or mitogenic stimulation. T-cell activation modifies the metabolic functions of T cells, including glucose metabolism, to meet the energetic demands of proliferation.[42] Thus, the inducible IL-10 production by $CD4^{LV\text{-}IL\text{-}10}$ T cells may depend on the activation of the LV-PGK promoter induced by glucose metabolism changes occurring in activated transduced T cells. However, we cannot exclude that the upregulation of IL-10 production by activated $CD4^{LV\text{-}IL\text{-}10}$ T cells is also due to the increased transcription of the endogenous IL-10 gene. In addition to the acquired ability to secrete high levels of IL-10, $CD4^{LV\text{-}IL\text{-}10}$ T cells lose the potential to produce IL-4, indicating that stable and constitutive over-expression of IL-10 by itself in unpolarized CD4+ T cells is sufficient to inhibit IL-4 production.

$CD4^{LV\text{-}IL\text{-}10}$ T cells upregulate IL-10–related markers such as PD-1, IL-10R, ICOS, ICOS-L, and HLA-G compared to $CD4^{LV\text{-}GFP}$ T cells. The variability in the expression of the IL-10–related markers in the different $CD4^{LV\text{-}IL\text{-}10}$ T cell lines was observed among different donors. This might be due also to the fact at the time of

the analysis the activation state of the cells was not homogeneous. Previous reports indicate that peripheral blood IL-10–producing Tr1-like cells express PD-1[36] and ICOS.[37] We recently demonstrated that *in vitro* differentiated Tr1 cells upregulate HLA-G.[24] The data obtained with $CD4^{LV\text{-}IL\text{-}10}$ T cells not only confirm these findings but also reinforce the notion that the production of IL-10 is sufficient for the acquisition of the Tr1 phenotype.

Like Tr1 cells,[8,12] $CD4^{LV\text{-}IL\text{-}10}$ T cells are anergic, suppress allogeneic $CD4^+$ and $CD8^+$ T cell responses mainly *via* IL-10 and TGF-β1, express high levels of GZ-B and acquired the ability to specifically lyse cells of myeloid origin. These results confirm the mode of action of Tr1 cells *in vitro*. The ability of $CD4^{LV\text{-}IL\text{-}10}$ T cells to control xeno-GvHD *in vivo* is the first demonstration in preclinical studies that human IL-10–producing Tr1 cells can prevent GvHD and inhibit disease progression. This protective effect is cell-dose dependent and requires a 1:1 ratio between $CD4^{LV\text{-}IL\text{-}10}$ T cells and effector T cells. In peripheral blood of mice cotransferred with effector $CD4^+$ T cells and $CD4^{LV\text{-}IL\text{-}10}$ or $CD4^{LV\text{-}GFP}$ T cells, GFP cells represented only a small fraction of the total $CD45^+CD4^+$ during the first 2 weeks (<10%), which declined in frequency over time as human chimerism increased (data not shown). Nevertheless, $CD4^{LV\text{-}IL\text{-}10}$ T cells prevented and inhibited GvHD progression through a mechanism, which is not yet completely elucidated.

Of notice, re-directing already differentiated $CD4^+$ T cells into Tr1 cells by means of LV-IL-10 requires long-term exposure to endogenous IL-10 with multiple rounds of T-cell division, suggesting that almost any $CD4^+$ T cell is able to convert into Tr1 in terms of phenotype and function after long-term exposure to autologous IL-10. This is in line with previous studies, where chronic exposure to antigen stimulation in the presence of IL-10 can induce Tr1 cells *in vivo*.[43] Interestingly, the acquisition of Tr1 cell characteristics is not related to naive or memory phenotype, as preliminary data suggest that both cell subsets become Tr1 cells upon LV-IL-10 transduction.

$CD4^{LV\text{-}IL\text{-}10}$ T cells are stable after long-term *in vitro* culture thereby upon *in vivo* infusion we foresee that LV-IL-10–transduced $CD4^+$ T cells will remain IL-10 producers. However, one concern regarding the future clinical use of LV-IL-10 transduced T cells is the fact that high levels of systemic IL-10 could lead to viral persistence.[44,45] Taken into account that, similar to Tr1 cells, LV-IL-10–transduced T cells produce significant amounts of IL-10 only after TCR activation, we anticipate that they could interfere with the natural outcome of a viral infection only in case this LV-IL-10 transduced T cells are specific for a viral antigen. It should be noted that in tolerant patients with high frequency of Tr1 cells and in patients adoptively transferred with allo-reactive Tr1 cells no systemic immunosuppression and normal immune responses to pathogens were detected ([12,17] and Bacchetta *et al.*, submitted).

IL-10 gene transfer could be applied to obtain antigen-specific Tr1 cells. We plan to generate antigen-specific $CD4^{LV\text{-}IL\text{-}10}$ T cells by transducing $CD4^+$ T cells specific for allo-antigens isolated *ex vivo* or generated *in vitro*. We will determine whether the resulting antigen-specific, IL-10-transduced $CD4^+$ T cell population can control responses in an antigen-specific manner without hindering the natural immunity of the host *i.e.,* by nonantigen–specific IL-10 (over)production. This approach might have significant advantages over the use of polyclonal regulatory T cells, which are currently applied in treating GvHD.[46]

In conclusion, we provide proof-of-principle that IL-10 overexpression in polyclonally activated human $CD4^+$ T cells is feasible and effective in re-directing T cells towards the Tr1 subset. Translating the LV-IL-10 approach to the clinic would require the use of a different marker gene other than GFP, possibly in combination with a suicide gene.[47] Furthermore, a targeted approach would include the generation of antigen-specific IL-10–transduced cells, to be used as Tr1-cell based therapy in organ or hematopoietic stem cell transplantation to prevent rejection or GvHD. A more challenging task will be to generate autoantigen-specific Tr1 cells by stimulating T cells with autologous DCs presenting the immunodominant self-antigen before transduction with LV-IL-10/GFP for treating autoimmune diseases.

Overall, result from this study provide strong evidence that genetic modification of human $CD4^+$ T cells is a feasible and promising approach to generate Tr1 cells for clinical use.

MATERIALS AND METHODS

Plasmid construction. The coding sequence of hIL-10 was excised from pH15C (ATCC n° 68192) by BamHI digestion and amplified with primers containing the restriction sites for EcoRI (5′) and XhoI (3′). The resulting 549 bp fragment was cloned into the multiple cloning site of pBluKSM (Invitrogen, Carlsbad, CA) previously digested with the above-mentioned restriction enzymes to obtain pBluKSM-hIL-10. A fragment of 555 bp was obtained by pBluKSM-hIL-10 digestion with XmaI and XhoI and ligated to pCCL.sin.cPPT.polyA.CTE.eGFP.minhCMV.hPGK.deltaNGFR.Wpre[35] (here named LV-GFP), previously digested with XmaI and SalI, to obtain LV-IL-10/GFP (here named LV-IL-10). The presence of the bidirectional promoter (human PGK promoter plus minimal core element of the CMV promoter in opposite direction) allows coexpression of the two transgenes. The sequence of LV-IL-10/GFP was verified by pyrosequencing (Primm). LV-GFP was used as control vector.

Vector production and titration. VSV-G–pseudotyped third generation LVs were produced by Ca_3PO_4 transient four-plasmid cotransfection into 293T cells and concentrated by ultracentrifugation as described[48] with a small modification: 1 µmol/l sodium butyrate was added to the cultures for vector collection. Titer was estimated on HeLa cells by limiting dilution, and vector particles were measured by HIV-1 Gag p24 antigen immunocapture (NEN Life Science Products, Waltham, MA). Vector infectivity was calculated as the ratio between titer and particle. For concentrated vectors, titers ranged from 5×10^8 to 6×10^9 transducing units/ml, and infectivity from 5×10^4 to 5×10^5 transducing units/ng of p24.

Cell preparation. Human peripheral blood was obtained from healthy donors upon informed consent in accordance with local ethical committee approval (Protocol PERIBLOOD) and with the Declaration of Helsinki. PBMCs were prepared by centrifugation over Ficoll-Hypaque gradients. $CD4^+$ T cells were purified by negative selection with the CD4 T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) with a resulting purity of >95%.

Transduction of human $CD4^+$ T cells. CD4-purified T cells were activated for 48 hours with soluble anti-CD3 mAb (30 ng/ml, OKT3; Janssen-Cilag, Raritan, NJ, USA), anti-CD28 mAb (1 µg/ml; BD Biosciences, San Diego, CA) and rhIL-2 (50 U/ml; Chiron, Milan, Italy). T cells were transduced with LV-GFP ($CD4^{LV\text{-}GFP}$) or LV-IL-10/GFP ($CD4^{LV\text{-}IL\text{-}10}$) with multiplicity of infection of 20. Transduced $CD4^+GFP^+$ T cells were purified 14 days after transduction by fluorescence-activated cell sorting and expanded in X-VIVO

15 medium supplemented with 5% human serum (BioWhittaker-Lonza, Washington, DC), 100 U/ml penicillin–streptomycin (BioWhittaker), and 50 U/ml rhIL-2. $CD4^{LV\text{-}GFP}$ and $CD4^{LV\text{-}IL\text{-}10}$ T cells were stimulated every 2 weeks in the presence of an allogeneic feeder mixture containing 10^6 PBMCs (irradiated 6,000 rad) per ml, 10^5 JY cells (an Epstein–Barr virus–transformed lymphoblastoid cell line expressing high levels of human leukocyte antigen and costimulatory molecules, irradiated 10,000 rad) per ml, and soluble anti-CD3 mAb (1 µg/ml). Cultures were maintained in X-VIVO 15 medium supplemented with 5% human serum, 100 U/ml penicillin–streptomycin, and 50–100 U/ml rhIL-2. All fluorescence-activated cell sorting phenotypic analysis, *in vitro* and *in vivo* experiments were performed in cells from at least 12 days after feeder addition, in resting state.

Vector copy number analysis. Cells were cultured for at least 14 days after transduction in order to get rid of nonintegrated vector forms. Genomic DNA was extracted from cells and purified using the QIAGEN blood and cell culture DNA kit (QIAGEN, Valencia, CA), according to manufacturer's instructions. Vector copy numbers per genome were quantified by quantitative PCR as described.[49] Copies per genome were calculated by the formula: (ng LV/ng endogenous DNA) × (n° of LV integrations in the standard curve). The standard curve was generated by using a CEM cell line stably carrying four vector integrants. All reactions were carried in out in triplicate in an ABI Prism 7900HT (Applied Biosystems, Foster City, CA).

T-cell proliferation and anergy assessment. $CD4^+$ T cells (2×10^5/well) were stimulated with immobilized anti-CD3 mAb (10 µg/ml) and soluble anti-CD28 mAb (1 µg/ml) in the presence or absence of rhIL-2 (100 U/ml) in complete X-VIVO medium in a final volume of 200 µl of medium in 96-well round-bottom plates. After 72 hours, cells were pulsed with 1 µCi/well ^{3}H-thymidine for additional 16 hours. To ensure the anergic state of LV-IL-10–transduced cells, (1×10^5) $CD4^{LV\text{-}IL\text{-}10}$ and control, $CD4^{LV\text{-}GFP}$ T cells, labeled with Vybrant/eFluor670, were stimulated with immobilized anti-CD3 mAb (10 µg/ml) and soluble anti-CD28 mAb (1 µg/ml) in the absence or presence of unlabeled PBMCs at increasing ratios (50–400 × 10^3–[K]). Proliferation was evaluated on GFP^+ cells measuring Vybrant/eFluor670 dilution by flow cytometry.

Suppression assays. To test the suppressive capacity of LV-transduced $CD4^+$ T cells, allogeneic purified $CD4^+$ or $CD8^+$ T cells were labeled with Vybrant DID or eFluor670 (Invitrogen) before stimulation with immobilized anti-CD3 (10 µg/ml) and soluble anti-CD28 (1 µg/ml) mAbs. Suppressor cells were added at 1:1 ratio. After 4–6 days of culture, proliferation of Vybrant/eFluor670-labeled $CD4^+$ or $CD8^+$ responder T cells was determined by flow cytometry after gating on $CD4^+GFP^-$ or $CD8^+CD4^-$ cells, respectively. In some experiments, antibodies against the IL-10R (clone CDw210, BD) and TGF-β1,2,3 (R&D, Minneapolis, MN) were concomitantly used at 50 µg/ml each. Transwell experiments were performed as previously described.[18]

Cytokine profile determination. To measure cytokine production, $CD4^{LV\text{-}GFP}$ and $CD4^{LV\text{-}IL\text{-}10}$ T resulting cells were stimulated with immobilized anti-CD3 mAb (10 µg/ml) and soluble anti-CD28 mAb (1 µg/ml) in the presence or absence of rhIL-2 (100 U/ml) or with leukocyte activation kit according to manufacturers' instructions (BD Pharmingen, San Diego, CA) in complete X-VIVO medium and in a final volume of 200 µl of medium (96-well round-bottom plates, 2×10^5/well). Brefeldin A was added during the last 2–3 hours of stimulation and cells were stained intracellularly following a 0.05% saponin protocol. In brief, after a 2.4G2 blocking step, cells were stained for surface markers, fixed with 2% paraformaldehyde for 15 minutes on ice and permeabilized for 30 minutes with 0.05% saponin/2% fetal bovine serum/phosphate-buffered saline. Next, cells were stained for IL-10 in the same permeabilizing solution, washed and immediately acquired on LSRII flow cytometer (Becton Dickinson). Culture supernatants were harvested after 24 and 48 hours of culture and levels of IL-2, IL-4, IL-5, IL-10, IFN-γ, TGF-β1and tumor necrosis factor-α were determined by capture enzyme-linked immunosorbent assay according to the manufacturer's instructions (BD Biosciences) or by Bioplex according to the manufacturer's instructions (Bio-Rad, Hercules, CA).

Flow cytometry analysis. For the detection of cell surface antigens, $CD4^{LV\text{-}GFP}$ and $CD4^{LV\text{-}IL\text{-}10}$ T cells were stained with mAbs against CD4, CD25, CD40, CD45RO, CD45RA, CD62L, CD127, CD137, CD161, IL-10R, ICOS, ICOS-L, PD-1, and HLA-G (Pharmingen-BD, eBioscience (San Diego, CA), AbCam (Cambridge, MA) or Biolegend (San Diego, CA)) after a 2.4G2 blocking step. Cells were incubated with the aforementioned Abs for 30 minutes at 4 °C in phosphate-buffered saline 2% fetal calf serum, washed twice and fixed with 0.2% formaldehyde. For the expression of GZ-A, GZ-B (BD Bioscience and Pharmingen) and FOXP3 (Biolegend), after surface staining, cells were fixed, permeabilized and stained with the FOXP3 staining kit according to the manufacturer's instructions (Biolegend).

Xeno-GvHD model. Eight to ten-week-old female NOD/scid mice were obtained from Charles-River Italia (Calco, Italy). The experimental protocol was approved by the internal committee for animal studies of our institution (Institutional Animal Care and Use Committee (IACUC)). The day before the cell transfer, mice were given 1 mg blocking anti-mouse CD122 (IL-2R-β) mAb intraperitoneally to neutralize residual natural killer activity.[50] The antibody was produced from the TMβ-1 hybridoma kindly provided by Professor Tanaka (Osaka University, Japan). At day 0, mice received total body irradiation with a single dose of 350 cGy ((γ) irradiation from a linear accelerator) and were immediately infused with negatively selected CD4 T cells alone (5×10^6) or with $CD4^{LV\text{-}GFP}$ or $CD4^{LV\text{-}IL\text{-}10}$ T cells (5×10^6 or 1×10^6). Cells were re-suspended in 500 µl of IMDM (Iscove's modified Dulbecco's medium) medium and infused intraperitoneally. Survival and weight loss was monitored at least three times per week as previously described[38] and moribund mice were humanely killed for ethical reasons. At weekly intervals, mice were bled and human chimerism was determined by calculating the frequency on human $CD45^+$ cells within the total lymphocyte population.

Cytotoxicity assays. The cytotoxic activity of $CD4^{LV\text{-}GFP}$ and $CD4^{LV\text{-}IL\text{-}10}$ T cells was analyzed in a standard ^{51}Cr-release assay. Briefly, 10^3 ^{51}Cr-labeled (NEN Dupont, Milan, Italy) target U937 or K562 cells were incubated for 4 hours with $CD4^{LV\text{-}GFP}$ or $CD4^{LV\text{-}IL\text{-}10}$ T cell lines at various effector-target cell ratios, plated in duplicate. Subsequently, the supernatant was removed and counted on a γcounter. Percentage of specific lysis was calculated according to the formula: 100 × (^{51}Cr experimental release – spontaneous release)/(maximum release – spontaneous release). Samples were tested in duplicate. T-cell degranulation was evaluated in a CD107a flow cytometric assay, according to the protocol described in ref. 14.

Statistical analysis. Numbers indicate mean ± SE. All statistical analyses for significant differences were performed with the two-tailed Mann–Whitney test. Survival was analyzed with the log-rank test. *P* values <0.05 were considered significant.

SUPPLEMENTARY MATERIAL

Figure S1. LV-IL-10 transduced $CD4^+$ T cells display predominantly central memory phenotype.
Figure S2. Similar levels of FOXP3 expression in LV-IL-10 and LV-GFP–transduced $CD4^+$ T cells.
Figure S3. IL-10R mRNA is expressed at high levels in LV-IL-10–transduced $CD4^+$ T cells.
Figure S4. Expression of the transcription factors T-bet and Gata-3 by LV-IL-10/GFP–transduced $CD4^+$ T cells.
Figure S5. $CD4^{LV\text{-}IL\text{-}10}$ T cell suppress $CD4^+$ T cell responses *via* IL-10 and TGF-β and in transwell.
Materials and methods.

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