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Elongation factor 1 (EF1α) promoter in a lentiviral backbone improves expression of the CD20 suicide gene in primary T lymphocytes allowing efficient rituximab-mediated lysis

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A B S T R A C T

Background and Objectives. CD20 has been proposed as a novel suicide gene system for the treatment of graft-versus-host disease (GVHD), a fatal complication of allogeneic bone marrow transplantation: indeed expression of the human non-immunogenic exogenous CD20 protein allows positive immunoselection of transduced cells as well as their killing *in vitro* with rituximab. Lentiviral vectors are promising tools in the field of gene therapy. We therefore searched for a lentivector giving good efficiency of transduction of human T lymphocytes activated by the sole addition of interleukin (IL)-2 and high expression levels of the CD20 transgene.

Design and Methods. The T-cell line CEM and peripheral T lymphocytes activated by phytohemagglutinin (PHA) and/or IL-2 were transduced with two different vectors carrying the CD20 transgene driven by either the phosphoglycerate kinase (PGK) or elongation factor 1α (EF1α) promoter, and using different multiplicities of infection (MOIs).

Results. Both the PGK- and EF1α-CD20 vectors allowed efficient transduction of the CEM cell line and PHA-activated T cells, reaching 99% and 90% in the different targets, respectively. However EF1α-CD20 led to much higher expression levels of the transgene (mean fluorescence intensity 588-618 compared to 53 for PGK-CD20). Furthermore lymphocytes activated with IL-2 alone could be efficiently transduced with EF1α-CD20, reaching 10-25% positivity for CD20 (mean fluorescence intensity 409-424), allowing adequate immunoselection and strong complement-mediated lysis.

Interpretation and Conclusions. EF1α-CD20 may represent a good candidate vector for gene therapy with the CD20 suicide system in the setting of allogeneic bone marrow transplants.

Key words: CD20, lentivirus, GVHD.

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Allogeneic bone marrow transplantation can cure several hematologic neoplasms, although its toxicity severely restricts its application.[1-3] In particular, the extremely active immune recognition of leukemic cells by donor's T lymphocytes (graft-versus-leukemia, GVL effect)[1,4-7] is counterbalanced by the life-threatening immune recognition of the host's normal cells by the same T cells (graft-versus-host disease, GVHD).[1,2,8-10] Recently, a genetic approach based on the introduction of suicide genes into the donor's T lymphocytes has been suggested to resolve this problem.[11-19]

We have previously described the development of an alternative idea based on the transduction of normal human CD20 cDNA into T lymphocytes using a Moloney-derived retroviral vector.[20-22] The normal human CD20 molecule can function both as a selection marker to immunopurify the transduced cells[22] and a killer gene following chimeric monoclonal anti-CD20 antibody rituximab administration.[23-27] Finally, the CD20 molecule should not be recognized as a foreign antigen. One of the most critical constraints of gene transfer into T lymphocytes is the requirement for T-cell receptor (TCR) activation and sustained proliferation in order to achieve retroviral vector transduction.[28] In fact, the culture system necessary to obtain an efficient level of transduction by Moloney-derived vectors may impair the immune competence of transduced cells, reducing the naïve T-cell subsets,[29] skewing the TCR repertoire[30] and decreasing alloreactivity.[31-33] Lentiviral vectors represent a good option to engineer T lymphocytes *ex vivo* without the need to induce a massive cell proliferation.[33-39] In order to ensure sufficient transgene expression in T lymphocytes using solely IL-2 activation, two lentiviral vectors

were constructed to compare the efficiency of the phosphoglycerate kinase (PGK) promoter[40] *versus* the elongation factor 1α (EF1α) promoter[41] in driving the expression of the CD20 suicide gene.

Design and Methods

Construction of CD20-lentiviral plasmids

We generated the pRRLsin.hPGK.CD20.Wpre (hereafter called PGK-CD20) and the pRRLsin.hEF1α.CD20.Wpre (hereafter called EF1α-CD20) by insertion of a 913-nucleotide fragment containing the complete coding sequence of the human CD20 cDNA.[21]

The CD20 cDNA was obtained by BamHI/SalI digestion of pGEMCD20 (a pGEM vector containing the full length human CD20 cDNA obtained by polymerase chain reaction amplification) and was subcloned into the BamHI/SalI sites in the pRRLsin.hPGK. EGFP.Wpre and pRRLsin.hEF1α.EGFP.Wpre[40,41] after removing the 846-bp fragment including the enhanced green fluorescent protein (EGFP) cDNA.

Production of CD20-vectors

HIV-derived vectors pseudotyped with the vesicular stomatitis virus (VSV) G envelope protein were produced by transient co-transfection of the 3 plasmids pMD.G, pCMVΔR 8.74 and CD20 transfer vector plasmid into the 293T epithelial cell line as previously described.[34]

Fresh supernatants were collected 48 hours after transfection and either frozen at –80° C or used immediately. Alternatively the supernatant was harvested, concentrated by ultracentrifugation, and resuspended in PBS-BSA. Concentrated viral stocks, treated with DNAase I, were stored at –80°C and titrated.[40]

Viral supernatant titer

The viral supernatant titers were evaluated by infecting a known number of CEM cells with different volumes of lentiviral supernatant as described previously.[34,42] The volumes of supernatants used (5 μL-1 mL for collected supernatant and 1 μL-30 μL for ultracentrifuged supernatant) were plotted against the percentages of CD20 positive target cells. The titer was calculated from the linear slope of the curve according to the formula: viral titer = (CEM cell number × percentage of fluorescent cells)/volume of supernatant and is expressed as CEM transducing units (T.U.) per milliliter (mL). Titers varied from 5×10^5 to 10^6 CEM T.U./mL for collected supernatant. The titer of the ultrastock preparation was 5×10^6 CEM T.U./μL.

Transduction of the CEM cell line

CEM cells were seeded in 12–well flat plates at 0.5×10^5 cells per well in 1 mL of RPMI 1640 (Seromed, Berlin, Germany) with 10% heat-inactivated FCS (Hyclone, Logan, UH, USA), glutamine and antibiotics (hereafter called complete RPMI), spun for 5 minutes at 1500 rpm to remove medium and then infected with one spin infection or four spin infections on two consecutive days as described above for T lymphocytes.

Transduction of human primary T cells with CD20-lentiviral vectors

Purified T lymphocytes were routinely prepared by Ficoll-Hypaque gradient separation (Lymphoprep Nycomed, Pharma, Oslo, Norway) and Percoll gradient (Amersham Biosciences, Uppsala, Sweden) centrifugation, as previously described.[34] The cells were seeded in 12-well flat plates at 1×10^6 cells per well in 1 mL of complete RPMI in the presence of 1 μg/mL PHA and 100 U/mL human recombinant (hr) IL-2 (Serono, Rome, Italy), or alternatively, in 100 U/mL hrIL-2 alone, for 48 hours before infection. On the second day 0.5×10^6 cells per well were plated in 12-well flat bottom plates and spun for 5 min at 1500 rpm to remove medium and then spin infected for 45 min at 1800 rpm with 1 mL of fresh or frozen lentiviral supernatants at a multiplicity of infection (MOI) of 0.15, 0.30, 0.50, and 1 CEM-T.U. in the presence of polybrene (8 μg/mL). After centrifugation, the medium was replaced with complete RPMI containing hrIL-2 (100 U/mL). In some experiments, this procedure was repeated 8 hours later and twice on the following day. The T cells were transduced with ultracentrifuged lentiviral supernatants by overnight exposure to titrated supernatants at MOI of 20 CEM-T.U. per cell in 12-well plates, in a final volume of 1 mL of complete RPMI with hrIL-2 (100 U/mL). On day 5 after the overnight infection, IL-2-activated cells were collected, centrifuged to remove medium and resuspended in 1 mL Clinimacs PBS/EDTA buffer (Clinimacs, Miltenyi Biotec, Bergisch Gladbach, Germany), labeled with anti-CD20 FITC (5 μL of antibody for every 1×10^6 CD20 positive cells) (BD Biosciences, San Josè, CA, USA) and subsequently with anti-FITC microbeads (10 μL of microbeads for every 1×10^6 total cells) (Miltenyi Biotec) and immunoselected with a separation column 25MS (Macs, Miltenyi Biotec) following the manufacturers' instructions. Cells were then washed and cultured in complete RPMI and hrIL-2 for 2 days (final concentration, 100 U/mL) before the complement-mediated lysis was assessed.

Analysis of phenotype

Flow cytometry analysis was performed 5 days after transduction: CD20 transduced T cells were detected using a standard labeling procedure with anti-CD20 FITC (clone L27, BD Biosciences) and anti-CD3 PE antibodies (clone Leu-4, BD Biosciences). All the samples were analyzed on a FACSCalibur (BD Biosciences) using CellQuest software.

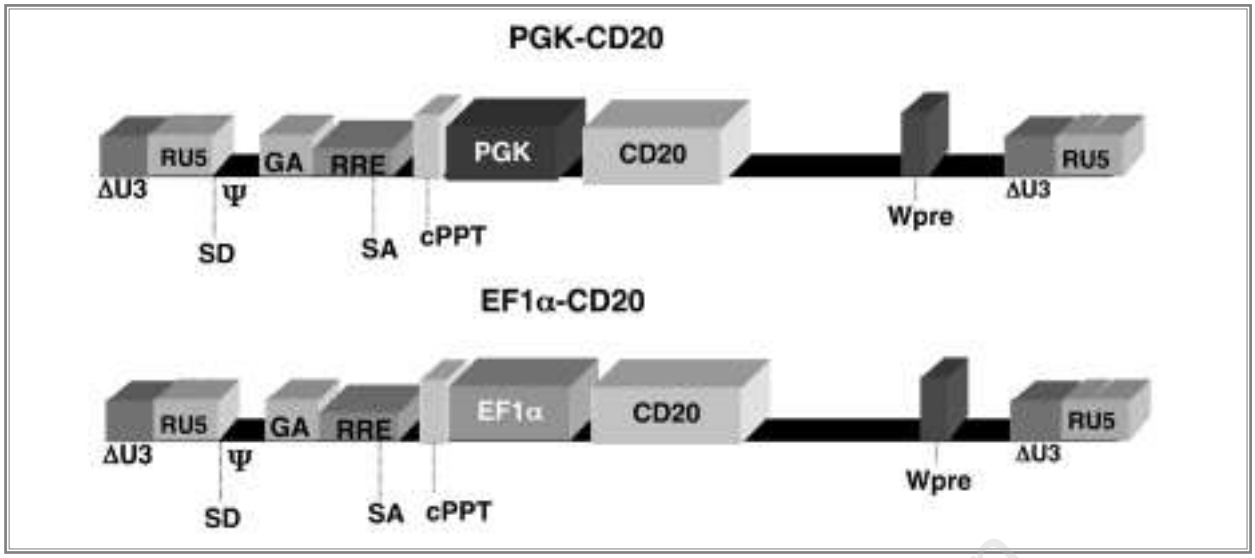

Figure 1. Schematic representation of the PGK-CD20 and EF1α-CD20 lentiviral vectors. The transduced *proviral* forms are shown. The vectors have an internal cassette carrying the human CD20 cDNA driven by the promoter of the human phosphoglycerate kinase gene (PGK) or by the human elongation factor 1α (EF1α). A 118-bp sequence encompassing the central polypurine tract is indicated as cPPT. The following viral *cis*-acting sequences are labeled: LTR regions (ΔU3, R, U5) with a deletion of 400 bp including the enhancer and promoter from U3, major splice donor site (SD); encapsidation signal (Ψ) including the 5' portion of the *gag* gene (GA); Rev-response element (RRE); splice acceptor sites (SA); and the post- transcriptional regulatory element of woodchuck hepatitis virus (Wpre).

Complement lysis assay

The complement cytotoxicity assay was performed essentially as already described[43,44] with some modifications. Briefly 50,000 cells/well were plated in 30 μL in triplicate in 96-well plates with 10 μg/mL rituximab in the presence or absence of rabbit serum, to a final concentration of 25%. The cells were incubated for 4 hours at 37°C, then diluted with medium to 270 μL and 1/10 volume of Alamar blue solution added (Biosource International, Camarillo, CA, USA). The plates were incubated overnight at 37°C and then read in a fluorimeter (Cytofluor 2300, Millipore, Bedford, MA, USA) with excitation at 530 nm and emission at 590 nm. In all cases, the effect of rituximab alone, in the absence of rabbit serum, was determined. The samples with rabbit serum alone were used as negative controls to normalize for the quenching of fluorescence by serum proteins and for the presence of dead cells in the samples in absence of any treatment. According to the manufacturer's instructions (Biosource), 0.25% Triton-X100 (Sigma, Saint Louis, MI, USA) was added to the wells used to set up the background fluorescence (all cells lysed). The same assay was performed on 3 samples to confirm the validity of the data.

Results

Construction of PGK-CD20 and EF1α-CD20 lentiviral vectors

Two lentiviral vectors were constructed to compare the efficiency of the PGK promoter (PGK-CD20, Figure 1) *versus* the EF1α promoter (EF1α CD20, Figure 1) in driving the expression of the CD20 suicide gene in T lymphocytes. The figure highlights the structural characteristic of the second generation lentiviral vectors, further optimized for promoting transgene expression by the introduction of the cPPT element from pol of HIV, encompassing the central polypurine tract and termination sequences,[40,45] by the presence of the post-transcriptional regulatory element of woodchuck hepatitis virus (Wpre) downstream of the CD20 stop codon[40] and for increased biosafety of the system by modification of the 3' LTR that has an almost complete deletion of the U3, thus being a self-inactivating vector (SIN).[46]

Transduction efficiency in the CEM cell line and PHA-activated T lymphocytes

To assess the effect of viral vector concentration on CD20 gene transfer efficiency, the CEM cell line was initially infected at different MOIs, with only one spin infection for 45 minutes at room temperature (Figure 2, panel A). Gene transfer efficiency was evaluated after 5 days as the percentage of CD20 positive cells by flow cytometric analysis for MOIs ranging from 0.15 to 1. As expected both vectors gave comparable percentages of transduced cells, varying from 11% to 38% for the different MOIs (Figure 2, panel A). In all cases, the EF1α-CD20 vector (black bars) showed higher expression of the CD20 transgene, with at least 5-fold increase in mean fluorescence intensity (MFI) over that shown by the PGK-CD20 vector (white bars) (Figure 2, panel B).

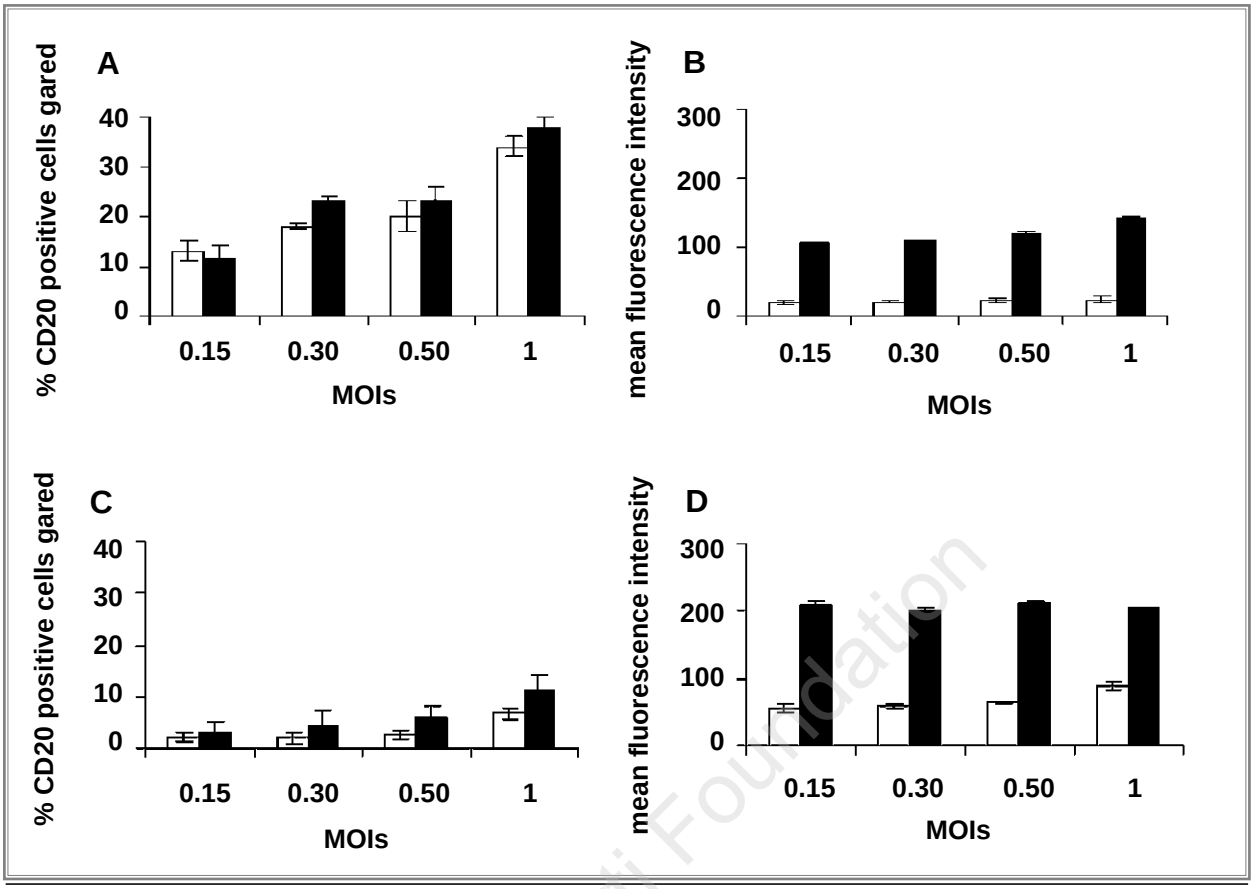

Figure 2. Transduction efficiencies of PGK-CD20 and EF1α-CD20 vectors following one spin infection. CEM cells (panel A and B) and PHA+IL-2 activated T cells were exposed to one spin infection at different MOIs of supernatants of PGK-CD20 (white bars) and EF1α-CD20 vector (black bars). FACS analysis of CEM cells and activated T lymphocytes revealed gene transfer efficiencies [shown as percentages of CD20-positive cells, panels A and C) and transgene expression levels (indicated as mean fluorescence intensity (MFI), panels B and D]. Data are representative of three sets of experiments.

To investigate whether this difference was also detectable in fresh cells, purified T lymphocytes were stimulated with media supplemented with PHA+IL-2 for 48 hours and subsequently exposed to PGK-CD20 and EF1α-CD20 vectors. As expected, the percentage of CD20 transduced cells was similar for both vectors, ranging from 2% to 7% for the PGK-CD20 vector and from 3% to 11% for the EF1α-CD20 vector (Figure 2, panel C). Similarly to what was observed with the CEM cell line, the EF1α-CD20 vector showed higher MFI values on PHA-activated T cells than did the PGK-CD20 vector, reaching a MFI value of 205 (± 5), with a 4-fold increase as compared to the PGK promoter (Figure 2, panel D). We then used the two CD20 lentiviral supernatants to transduce the CEM cell line and T-lymphocytes activated for 48 hours with PHA with a double spin infection for two consecutive days to maximize the cell transduction. As shown in Figure 3, most of the cells were then transduced (more than 94% for CEM, upper panels and more than 75% for T lymphocytes, lower panels). Nonetheless, the EF1α-CD20 vector still showed superior MFI values with respect to the PGK-CD20 vector both in the CEM cells (right upper panel) and in T lymphocytes (right lower panel).

Human T lymphocytes are efficiently transduced by EF1α-CD20 lentiviral vector in the absence of PHA stimulation

This first part of the experiments suggested that the EF1α-CD20 vector was the most suitable for further examination. We, therefore, cultured T cells for 48 hours in the presence of IL-2 and infected them with a titrated concentration (MOI 20) of EF1α-CD20 and overnight exposure.

The efficiency of transduction was also tested in parallel in T cells activated for 48 hours by PHA + IL-2. As shown in Figure 4, PHA stimulation resulted, as expected, in higher transduction efficiency (84% double pos-

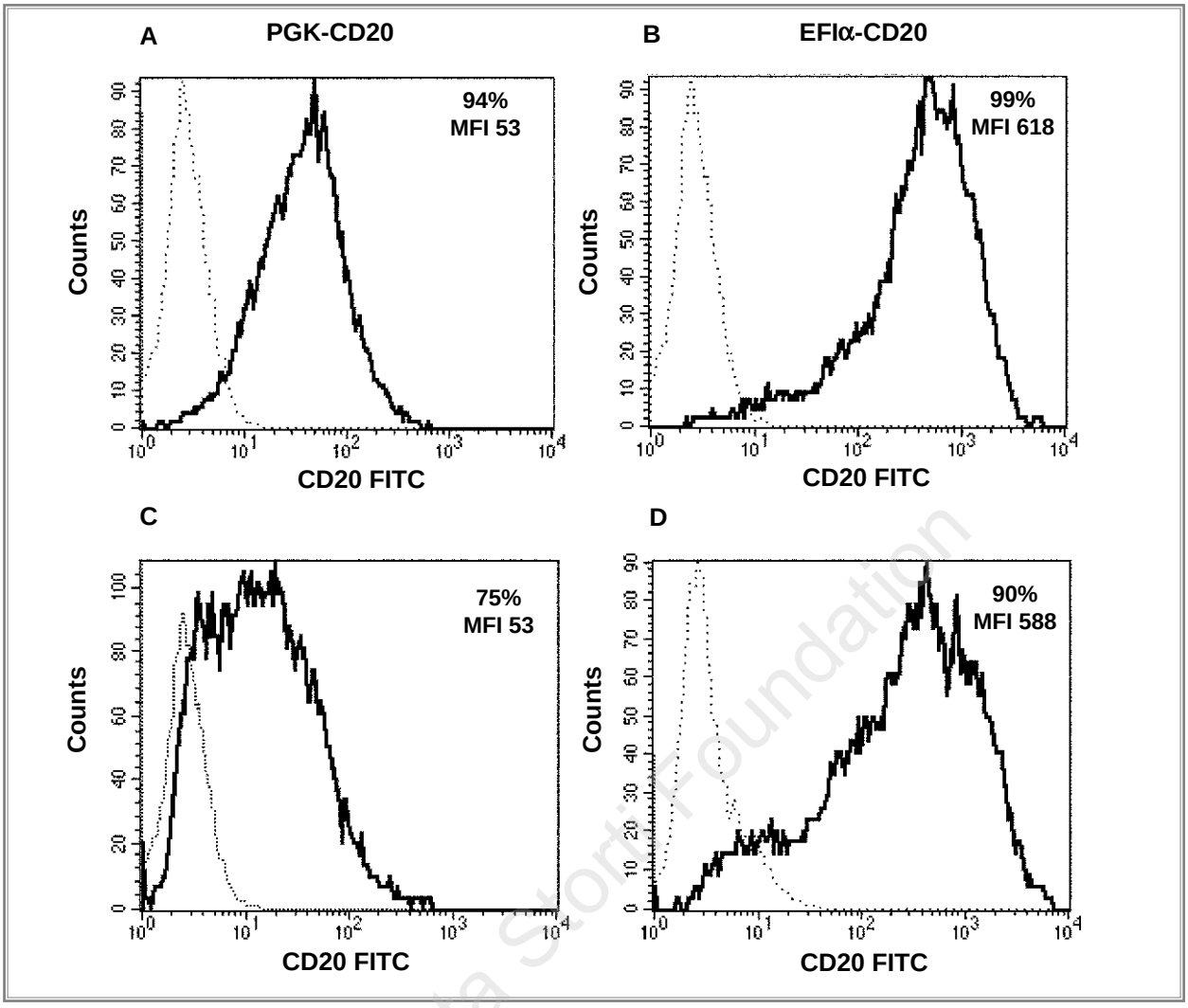

Figure 3. Transduction efficiencies of PGK-CD20 and EF1α-CD20 vectors following four spin infections. CEM cells (panels A and B) and T lymphocytes activated by PHA + IL-2 (panels C and D) were exposed to four separate spin infections on two consecutive days. Gene transfer efficiency was assessed 5 days later. The percentages of CD20-positive cells and the mean fluorescence intensity (MFI) are indicated in each panel. The corresponding profile for mock-transduced cells (dashed lines) is overlaid for comparison.

itive CD3/CD20 cells). However, IL-2 activation alone was sufficient for an efficient CD20 transduction, ranging from 10% to 25% of CD20 positive T cells in ten different donors (*data not shown*). In the representative experiment shown in Figure 4, 17% CD20 expression was achieved.

In order to verify the efficiency and reproducibility of immunoselection of IL-2 activated and CD20-transduced lymphocytes, a standard protocol for column immunoselection was tested on 3 consecutive samples. One representative example is shown in Figure 5, panel A and demonstrates the high purity of the CD3/CD20 positive sorted population (85% CD3/CD20 double positive selected cells).

IL-2-activated T lymphocytes transduced with the EF1α lentiviral vector are susceptible to rituximab-complement mediated lysis

In order to verify whether the CD20 transgene on the cell surface of IL-2-activated T lymphocytes renders the cells susceptible to rituximab-mediated cytotoxicity *in vitro* we carried out a complement lysis experiment in the presence or absence of rituximab on CD20-transduced T lymphocytes activated solely by IL-2, before and after immunoselection. A standard concentration of rabbit serum was added as a source of complement in the presence or absence of rituximab. Serum alone had no toxic effect. The percentage of cells killed by complement-dependent cytotoxicity equalled the percent-

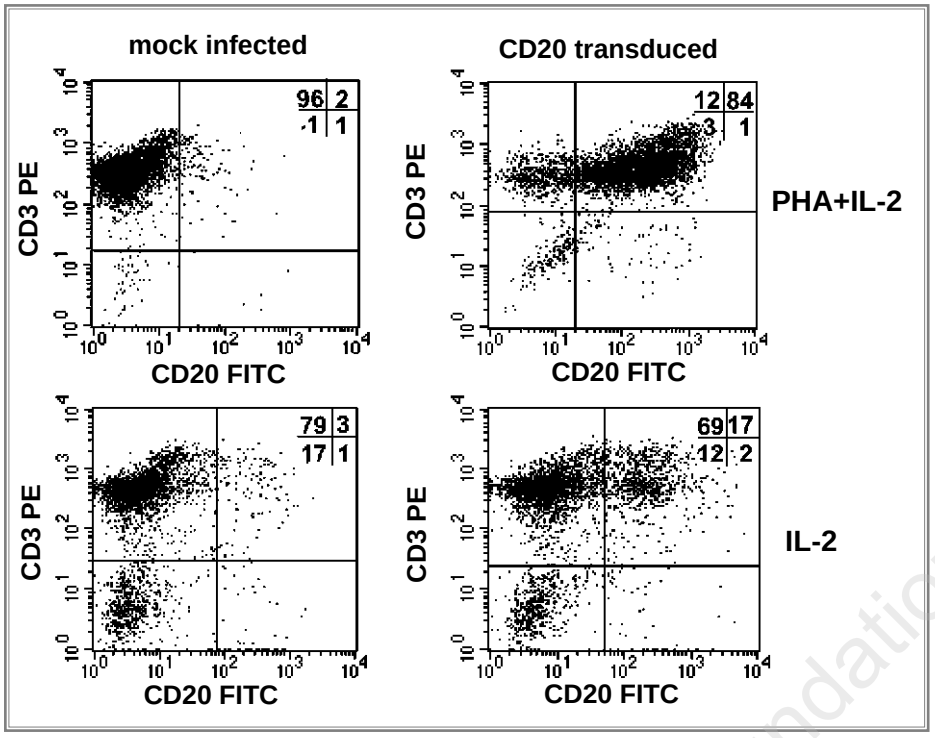

Figure 4. Flow cytometric analysis of CD20 expression in T lymphocytes activated by PHA + IL-2 or with IL-2 alone. PHA+IL-2 activated or IL-2-activated T lymphocytes were transduced by one overnight exposure to titrated stocks of ultracentrifuged EF1α-CD20 vector at a MOI of 20. Cells were stained with anti-CD20 FITC and anti-CD3 PE.

age of CD20 positive cells in both populations before and after selection (one representative experiment is shown in Figure 5, panel B).

The cells not lysed after the complement-dependent cytotoxicity assay were stained with anti-CD20 FITC antibody demonstrating the complete absence of CD20 positive cells in that population (*data not shown*). We ruled out the possibility that rituximab blocks the epitope recognized by anti-CD20 antibody since competition experiments performed earlier showed no interference (*data not shown*).

Discussion

Recently a novel strategy was developed in order to use the normal human CD20 molecule as a selection marker and as a target for rituximab-mediated lysis in the context of the development of suicide gene therapy approaches for the treatment of GVHD during allogeneic bone marrow transplantation.[21]

In this report we demonstrate that EF1α-CD20 lentiviral vector is more efficient than PGK-CD20 lentiviral vector for CD20 transgene expression in the human CEM T-cell line and in fresh T lymphocytes. Furthermore, we shown that EF1α-CD20 lentiviral vector can efficiently transduce T lymphocytes exposed to IL-2 alone and that CD20-transduced cells can be immunoselected and killed by rituximab complement-mediated lysis.

Since we previously described that rituximab-mediated lysis correlates with the level of expression of surface CD20 antigen,[44] it is important to develop new vectors to optimize the expression of the CD20 marker, rendering T cells more susceptible to rituximab-mediated lysis. To do this, we compared two lentiviral vectors differing only for their internal promoter. Since Salmon *et al.*[41] demonstrated that lentiviral vectors containing an internal EF1α promoter induce high levels of transgene expression in primary T cells, we decided to compare the relative abilities of PGK and EF1α-containing vectors to induce high levels of CD20 expression in T lymphocytes. The data obtained both at relative high and low MOI (to rule out the effect of multiple integration sites) strongly suggest that the EF1α promoter is indeed more efficient than the PGK in human T lymphocytes, as clearly indicated by the difference in MFI value.

We have previously described the efficient transduction of PHA-activated T cells by a Moloney-derived vector containing the CD20 gene.[21,22] One of the major limits of transduction by this kind of vector is that the target cells must be induced to proliferate.[47-50] It has been widely demonstrated that non-physiologic activation of T cells *in vitro* (for example, by PHA+IL-2) leads to the loss of certain memory T-cell subsets, such as those responsible for anti-EBV and CMV responses,[29,31,32,51] to skewing of the polyclonal T-cell receptor *versus* oligoclonality and to inversion of the CD4/CD8 ratio.[31,52] Newly developed lentiviral vectors represent the best choice because of their capacity to transduce and mediate long-term expression of transgenes into non-mitotic cells.[28,34,36,53] Recently Cavalieri *et al.*[33] showed that lentivi-

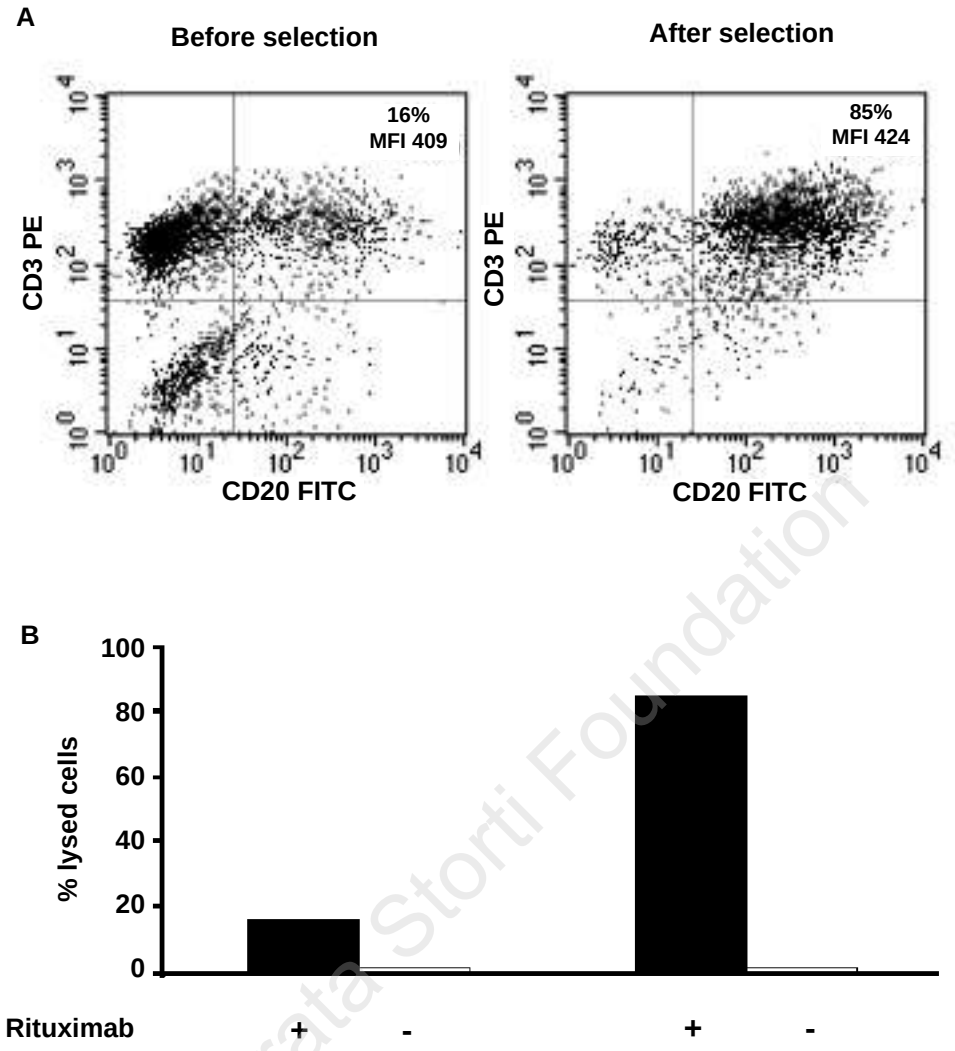

Figure 5. Efficiency of gene transduction, immunoselection and lysis of transduced IL-2-stimulated T lymphocytes. Panel A. Dot plot of a representative example of solely IL-2-activated CD20-transduced cells stained with anti-CD20 FITC and anti-CD3 PE, before (left) and after (right) immunoselection. Cells were incubated with anti-CD20 FITC and anti-FITC microbeads, then selected with a separation column. Numbers refer to the percentage of CD20+/CD3+ cells and the mean fluorescence intensity (MFI) Panel B. Complement-mediated lysis of the IL-2 stimulated cells before and after immunoselection. CD20-transduced unselected (left) or immunoselected (right) populations were lysed with rabbit serum as a source of complement either in the presence (+) or in the absence (–) of rituximab.

ral vectors allowed efficient transduction of T cells stimulated solely with IL-2, preserving most of the cells' functions.[33] In our set of experiments we were able to transduce a significant percentage of T cells with the EF1α-CD20 lentivector in culture systems with both PHA+IL2 and, more importantly, with only IL-2.

Several genes have so far been proposed for the suicide gene therapy approach to treat GVHD in bone marrow allografts.[11-19, 54] The best available model is constituted by the herpes simplex-derived thymidine kinase virus (TK) gene.[19,55] Unfortunately, several limitations have been observed during the clinical application of the TK system: 1) some patients develop an immune response against foreign TK protein[56] 2) patients may need ganciclovir treatment for concomitant infections experiencing an unwanted elimination of engineered cells,[19] 3) some patients showed *in vivo* selection of TK mutants due to the presence of a cryptic splice site in the gene.[57,58] Finally, non-dividing cells are not susceptible to ganciclovir-induced lysis.[56]

The CD20 gene system offers several advantages: 1) the CD20 complete human cDNA should not be anti-

genic; 2) the engineered cells should be killed exclusively by rituximab, a drug which is not usually used for the treatment of bone marrow transplant patients;[23-27] 3) cells are rapidly selected and the CD20 molecule does not code for enzymatic activity;[59] 4) finally CD20 expression should be maintained also in non-dividing cells, as documented for normal B lymphocytes.[60] Furthermore, the CD3/CD20 double positive T cells do not represent a completely artificial modification of the T lymphocytes, because a small, but detectable percentage of CD3/CD20 positive cells exists in normal donors as reported by Hultin *et al.*[61] and confirmed by the CD3/CD20 phenotype of the mock-infected population shown in Figure 4; the functional role of these double positive cells is obscure.[61-63,64]

In this study we made use of recently developed second generation lentiviral vectors additionally modified by a deletion in the U3 region of the LTR (for this reason called self-inactivating vectors, SIN) which renders them unable to transcribe from the LTR viral promoter, reducing the risk of recombination.[46,53,65,66] The development of safer vectors will be a crucial issue to favor clinical applications, also in consideration of the recently described risks of insertional mutagenesis, which occurred *in vivo* in animals and in patients.[67,68] One final important advantage of the CD20 molecule, from this point of view, lies in its molecular structure, which does not suggest any signaling function. Indeed our preliminary experiments with microchip technology seem to confirm this hypothesis (*Golay et al., unpublished data*). In conclusion, the data obtained using the EF1 α-CD20 lentivector should be useful for developing an innovative system for T-lymphocyte transduction which should produce more functional and immunologically preserved cells with bright expression of CD20.

Contributions. All authors provided substantial contributions to the conception and design of the study, acquisition of evidence, analysis and interpretation of the data. All authors also participated in drafting the article and revising it critically, and gave final approval of the version to be published. M.S. and M.B. were responsible for the conduction of the experiments, data collection and project's design. All authors were responsible for analysis of results and for manuscript preparation. The authors are indebted to Professor Jane Apperley, Department of Hematology, Imperial College School of Medicine, Hammersmith Hospital, London for critical suggestions and helpful discussions throughout the course of this work. The authors thank Professor Luigi Naldini, Institute for Cancer Research and Treatment, University of Turin, Candiolo, Italy and Professor Didier Trono, Faculty of Medicine, Geneva, Switzerland for providing vectors utilized in this work. The authors are grateful for the consistent and reliable technical support of Sergio Bernasconi, Giancarlo Bianchi and Gianmaria Borleri. The authors reported no conflict of interest.

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