# Retroviral transfer of human CD20 as a suicide gene for adoptive T-cell therapy

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## ABSTRACT

The aim of adoptive T-cell therapy of cancer is to selectively confer immunity against tumor cells. Autoimmune side effects, however, remain a risk, emphasizing the relevance of a suicide mechanism allowing *in vivo* elimination of infused T cells. We investigated the use of human CD20 as suicide gene in T-lymphocytes. Potential effects of forced CD20 expression on T-cell function were investigated by comparing CD20- and mock-transduced cytomegalovirus (CMV) specific T cells for cytolysis, cytokine release and proliferation. The use of CD20 as suicide gene was investigated in CMV specific T cells and in T cells genetically modified with an antigen specific T-cell receptor. No effect of CD20 on T-cell function was observed. CD20-transduced T cells with and without co-transferred T-cell receptor were efficiently eliminated by complement dependent cytotoxicity induced by therapeutic anti-CD20 antibody rituximab. The data support the broad value of CD20 as safety switch in adoptive T-cell therapy.

Key words: gene therapy, suicide gene, donor lymphocyte infusions, T-cell receptor.

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## Introduction

The aim of adoptive T-cell therapy of cancer is to selectively confer immunity against tumor cells. Adverse side effects, however, may occur and lead to severe autoimmunity or graft-versus-host disease (GvHD), emphasizing the relevance of a suicide mechanism that allows efficient *in vivo* elimination of infused T cells.

Herpes simplex virus thymidine kinase (HSV-tk) is a well-established suicide gene that has been successfully used to control GvHD following donor lymphocyte infusions (DLI) after allogeneic stem cell transplantation.[1,2] The anti-tumor effect of DLI is mediated by donor T cells recognizing allo-antigens on the malignant cells of the recipient. Donor T cells may also induce GvHD due to recognition of allo-antigens on non-malignant tissues of the patient. Transfer of HSV-tk to DLI preserves the beneficial anti-tumor effect and allows *in vivo* elimination of donor T cells if severe GvHD occurs.

T-cell receptor (TCR) gene transfer is an attractive strategy for rapid *in vitro* generation of high numbers of antigen specific T cells.[3] Autologous lymphocytes genetically modified with the TCR for MART-1/Melan-A have been adoptively transferred to patients with advanced melanoma.[4] In addition, TCRs for minor histocompatibility antigens, including HA-1 and HA-2, have been transferred to donor-derived virus-specific T cells to treat patients with hematologic malignancies after allogeneic stem cell transplantation.[5-8] A drawback of TCR gene therapy is the formation of mixed TCR dimers, consisting of transgenic and endogenous TCR α and β chains. Mixed TCR dimers have unknown specificities and may be potentially auto-reactive. Co-transfer of a suicide gene would provide a desirable safety switch in clinical TCR gene therapy.

Human CD20 has been proposed as non-immunogenic protein with a dual function as selection marker and suicide gene.[9,10] Rituximab (RTX) is a therapeutic anti-CD20 antibody, which is widely used in the clinic.[11-14] Upon ligation of CD20, RTX triggers various effector mechanisms, including complement-dependent cytotoxicity (CDC).[15] Several second generation anti-CD20 antibodies have recently been developed, and clinical trials are currently ongoing to investigate their *in vivo* efficacy.

Although human CD20 has previously been described as functional selection marker and suicide gene after retroviral transfer to T lymphocytes,[9,10] the specificities of these T cells were unknown, and therefore a potential effect of CD20 on antigen-specific T-cell functions could not be investigated. In this study, we investigated the use of CD20 as safety switch after retroviral transfer to T cells with different cytomegalovirus (CMV) specificities, and conclude that CD20 may be broadly applicable as safeguard in adoptive T-cell therapy.

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## Design and Methods

### Cell culture

Peripheral blood mononuclear cells (PBMC) were collected from healthy donors after approval by the LUMC institutional review board and informed consent according to the Declaration of Helsinki. EBV-transformed B cells (EBV-LCL) were cultured in IMDM (Cambrex, Verviers, Belgium) with 10% Fetal Bovine Serum (FBS). T-cell clones were stimulated every 10-14 days with irradiated allogeneic PBMC and phytohemagglutinin in IMDM with 5% human serum (HS), 5% FBS and 100 IU/mL IL-2 as previously described.[5,7]

### Construction of CD20-encoding retroviral vector

RNA was isolated from EBV-LCL using Trizol (Invitrogen, Breda, The Netherlands) and transcribed to cDNA using M-MLV reverse transcriptase (Invitrogen). PCR was performed using Expand High Fidelity Enzyme (Roche Diagnostics, Almere, The Netherlands) and primers as described by Introna *et al.*[6] The 898 bp CD20 cDNA was cloned into retroviral LZRS and MP71 vectors, which both contain the truncated human nerve growth factor receptor (NGFR) selection marker gene linked by an IRES sequence.[7,8]

### Production and transduction with retroviral vector supernatants

Retroviral vector supernatants were produced by transfecting packaging F-NX-A cells with retroviral LZRS and MP71 vectors as previously described.[7,8] $CD8^+$ cells were isolated from PBMC by magnetic beads (Miltenyi Biotec GmbH), and stimulated with CMV peptides (each at 1 μg/mL), containing HLA-A*0101-binding pp50 VTEHDTLLY (pp50-VTE) and HLA-B*0801-binding IE1 ELRRKMMYM (IE1-ELR) and QIKVRVDMV (IE1-QIK).[16,17] Peptide-stimulated $CD8^+$ cells were transduced with retroviral vector supernatants as previously described.[8] CD20-transduced T cells (5-10×$10^6$ cells) were incubated with PE-labeled anti-CD20 and isolated by anti-PE coated magnetic beads (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany).

### Tetrameric complexes and flow cytometric analysis

Tetramers were constructed for CMV pp50-VTE, IE1-ELR and IE1-QIK and HLA-A*0201 HA-2 YIGEVLVSM,[18] as previously described.[19] Cells were stained with APC- or PE-labeled tetramers for 2 h at 4°C in medium without phenol with 2% FBS. FITC-labeled anti-CD20 and APC- or PE-labeled anti-NGFR antibodies (Becton Dickinson, Breda, The Netherlands) were added during the final 30 min.

### ELISA

EBV-LCL were pulsed with titrated CMV peptides for 2 h at 37°C, washed and used as stimulator cells (3×$10^4$ cells/well) for CMV specific T cells (5×$10^3$ cells/well) in duplicate in 96-well plates. After 24 h of incubation at 37°C, IFN-γ release in 50 μL supernatants was measured by ELISA (Sanguin, Amsterdam, The Netherlands).

### $^{51}Cr$-release assay

EBV-LCL were labeled with 50 μCi $Na_2{}^{51}CrO_4$ for 60 min at 37°C, and directly used as target cells or after incubation with titrated CMV peptides for 1 h at 37°C. Target cells were seeded in 96-well plates together with CMV specific T cells. After 4 h of incubation, 25 μL of supernatant was harvested and measured as previously described.[7,8]

### Proliferation assay

CMV specific T cells were stained with 5 mM CFSE (Molecular Probes Europe, Leiden, The Netherlands) for 10 min at 37°C. CMV specific T cells (2×$10^4$ cells/well) were stimulated with EBV-LCL pulsed with titrated CMV peptides (1×$10^4$ cells/well) and irradiated allogeneic PBMC (1×$10^5$ cells/well) in triplicate in 96-well plates in IMDM with 5% HS, 5% FBS and 25 IU/mL IL-2. At day 3, cell cultures were stained with APC-labeled anti-NGFR and numbers of viable (PI-) cells were analyzed by flow cytometry. The proliferation (%) is the number of $NGFR^+$ cells with diluted CFSE of total $NGFR^+$ $CFSE^+$ cells x 100%.

### Complement dependent cytotoxicity

T cells (1×$10^4$ cells/well) were incubated in triplicate in 96-well plates in 50 μL IMDM with or without RTX (20 μg/mL; Hoffman-La Roche, Basel, Switzerland). After 20 min of incubation at RT, IMDM with baby rabbit complement (BRC, 1:8 working dilution, Cedarlane, Ontario, Canada) or 10% heat inactivated human serum (HS) was added to a final volume of 100 μL. After 1 h of incubation at 37°C, numbers of viable ($PI^-$) cells were analyzed by flow cytometry. Viability was calculated as the percentage of viable cells of 2×$10^4$ total events.

## Results and Discussion

### Delivery of human CD20 by retroviral vectors

To investigate CD20 expression after retroviral transduction, the CD20 cDNA was cloned into retroviral LZRS and MP71 vectors which also contained the truncated human nerve growth factor receptor (NGFR) selection marker gene (LZRS-CD20-IRES-NGFR and MP71-CD20-IRES-NGFR) and introduced into Jurkat-T cells. As shown in Figure 1, expression of the CD20 and NGFR genes was significantly higher after delivery by the MP71 vector as compared to the LZRS vector.

### Human CD20 as suicide gene in cytomegalovirus specific T cells

To investigate whether introduction of human CD20 leads to altered T-cell function(s), T cells with known specificities for CMV pp50-VTE, IE1-ELR and IE1-QIK were transduced with MP71-CD20-IRES-NGFR. $CD8^+$ cells isolated from PBMC from a healthy donor were stimulated with a mixture of CMV peptides and transduced with MP71-CD20-IRES-NGFR or empty vector (MP71-IRES-NGFR) at day 3. At day 10, transduced CMV-$tetramer^+$ cells were single cell sorted by flow cytometry. CD20- and mock-transduced CMV specific

T-cell clones were isolated (Figure 2A) and compared for lytic activity, cytokine release and proliferative capacity upon stimulation with autologous EBV-LCL pulsed with titrated CMV peptides (Figure 2B). The data show no evidence for impaired T-cell function upon introduction of human CD20. Next, the capacity of CD20 to function as suicide gene was investigated by measuring RTX-induced CDC. CD20- and mock-transduced CMV specific T cells were treated with RTX and BRC, or heat inactivated HS as a control, and analyzed for numbers of viable cells by flow cytometry as well as cytolytic activity against peptide-pulsed autologous EBV-LCL in $^{51}$Cr-release assays at effector to target (E:T) ratios of 10:1 as determined prior to CDC. Treatment with RTX and BRC strongly diminished viability as well as lytic activity (Figure 2C) of CD20-transduced, but not mock-transduced, T cells. The data show that CD20 functions as appropriate suicide gene after transfer to CMV specific T cells.

### *Human CD20 as suicide gene in TCR gene transfer*

The capacity of CD20 to function as suicide gene in TCR gene transfer was addressed by introducing separate HA-2 TCR- and CD20-encoding vectors into CMV specific T cells. CD8$^+$ cells from a healthy donor were stimulated with a mixture of CMV peptides (pp50-VTE, IE1-ELR and IE1-QIK) and transduced with MP71-CD20-IRES-NGFR at day 2. At day 9, the CD20-transduced T cells which were >90% CMV-tetramer$^+$ were isolated by anti-CD20 coated beads. CD20-isolated T cells rapidly proliferated and retained high purities of transduced cells after one week of *in vitro* expansion (Figure 3A), indicating that CD20 properly functions as selection marker. CD20-isolated T cells were subsequently restimulated and transduced with an HA-2 TCR-encoding vector (LZRS-AV15-IRES-BV18[8]) at day 2. At day 9, HA-2 tetramer+ CD20$^+$ NGFR$^+$ cells were sorted by flow cytometry. The sorted T cells rapidly expanded upon restimulation and remained highly purified for HA-2 tetramer$^+$ CD20$^+$ NGFR$^+$ cells (Figure 3B). The function of CD20 as suicide gene in *TCR* gene transfer was investigated by measuring RTX-induced CDC. T cells co-transduced with CD20 and HA-2 TCR, and a parental HA-2 specific CTL as a control, were treated with RTX and BRC or heat inactivated HS, and subsequently analyzed for cytolytic activity against HA-2 peptide-pulsed EBV-Z (HLA-A*0201$^+$ HA-2$^-$) and EBV-RZ (HLA-A*0201$^+$ HA-2$^+$) at E:T ratios of 10:1 as determined prior to CDC. Treatment with RTX and BRC strongly reduced the lytic activity of T cells transduced with CD20 and HA-2 TCR, whereas no effect on the parental

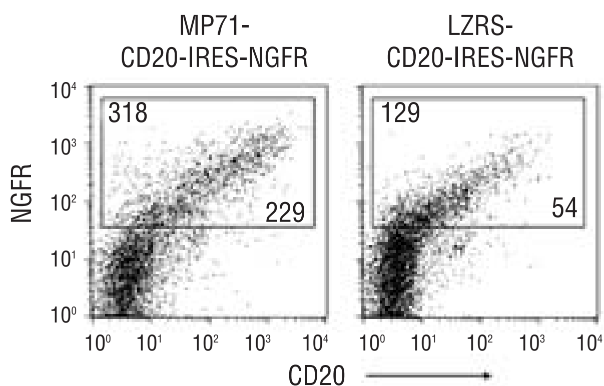


Figure 1. Delivery of human CD20 by retroviral vectors. Jurkat-T cells were transduced with MP71-CD20-IRES-NGFR and LZRS-CD20-IRES-NGFR retroviral vectors. Indicated are the mean fluorescence intensities after staining with PE-labeled anti-CD20 and APC-labeled anti-NGFR antibodies.

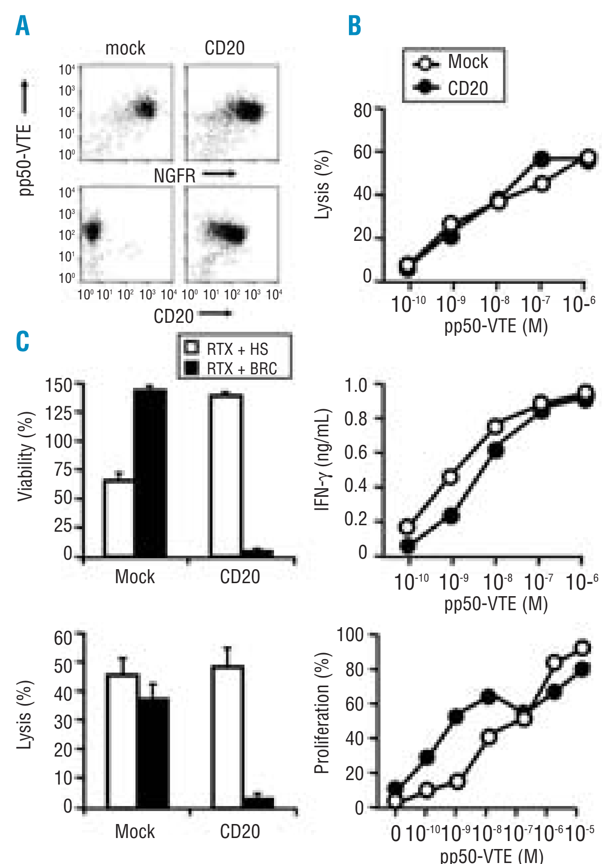


Figure 2. Human CD20 as suicide gene in cytomegalovirus specific T cells. CD20- and mock-transduced T-cell clones specific for pp50-VTE, IE1-ELR and IE1-QIK were isolated and compared for functional activity upon stimulation with autologous EBV-LCL pulsed with titrated CMV peptides. T cells specific for pp50-VTE are shown as representative examples. (A) Flow cytometric analysis after staining with CMV tetramers and antibodies against CD20 and NGFR. (B) T cells were tested for cytolytic activity in $^{51}$Cr-release assays at E:T ratios of 10:1 (upper), specific release of IFN-γ in ELISA (middle) and proliferative capacity by flow cytometry (lower). (C) CD20- and mock-transduced CMV specific T-cell clones were compared for RTX-induced CDC. T cells were treated with RTX and BRC or heat inactivated HS for 1 h at 37°C and analyzed for percentages of viable (PI-) cells relative to cultures incubated in medium with heat inactivated HS without RTX (100%) (upper). After CDC, T cells were washed and tested for cytolytic activity against peptide-pulsed autologous EBV-LCL in $^{51}$Cr-release assays at E:T ratios of 10:1 as counted prior to CDC (lower).

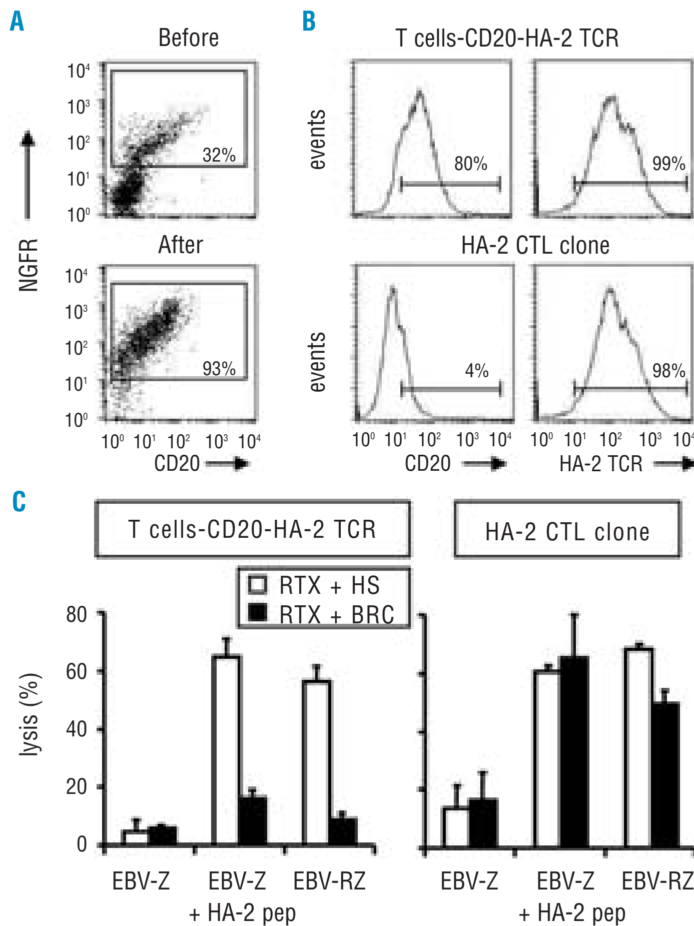


**Figure 3.** Human CD20 as suicide gene in TCR gene transfer. (A) CD20-transduced peptide-stimulated CD8+ cells from a healthy donor were analyzed by flow cytometry before (upper) as well as one week after (lower) isolation by magnetic beads and subsequent *in vitro* expansion. (B) CD20-isolated T cells were restimulated and transduced with the HA-2 TCR. CD20+ HA-2-tetramer+ T cells were sorted by flow cytometry. Indicated is the MFI of anti-CD20 and HA-2 tetramer staining one week after sorting (upper). As a control, MFI values are shown for a parental HA-2 specific CTL (lower). (C) T cells co-transduced with CD20 and HA-2 TCR (left) and the parental HA-2 specific CTL (right) were compared for RTX-induced CDC. T cells were treated with RTX and BRC or heat inactivated HS for 1 h at 37°C. After CDC, T cells were washed and analyzed for cytolytic activity against HA-2 peptide-pulsed EBV-Z (HLA-A*0201+ HA-2-) and EBV-RZ (HLA-A*0201+ HA-2+) in 51Cr-release assay at E:T ratios of 10:1 as determined prior to CDC.

HA-2 specific CTL was measured (Figure 3C). Treatment with RTX and heat inactivated HS did not diminish the survival and lytic activity of transduced T cells (*data not shown*). These data show that CD20 can be successfully co-transferred as suicide gene in *TCR* gene transfer.

Human CD20 has been proposed as a non-immunogenic protein with a dual function as selection marker and suicide gene.[9,10] We transferred the *CD20* gene to CMV specific T cells and performed a detailed investigation of a potential effect of CD20 on T-cell function. Our data show no evidence for impaired T-cell functions. We also demonstrate that CD20 can be used as efficient safety switch in CMV specific T cells and in T cells genetically modified with an antigen specific TCR. Although CD20 has been described as functional selection marker and suicide gene after retroviral transfer to T lymphocytes,[9,10] its use has been debated. A possible explanation for this controversy may be the choice of retroviral vector. We cloned the *CD20* gene into LZRS and MP71 vectors and observed a marked difference in surface expression, illustrating the importance of vector selection for appropriate transgene expression. CD20 expression as mediated by the MP71 vector allowed efficient isolation of CD20-transduced T cells, whereas CD20 expression as mediated by the LZRS vector may be below the threshold of a functional selection marker.

Clinical studies have demonstrated the successful use of HSV-tk as suicide gene in the control of GvHD in patients receiving DLI under conditions of profound immunosuppression.[1,2,20] In immunocompetent patients receiving HSV tk gene-modified DLI late after transplantation, however, gene-modified lymphocytes rapidly disappeared due to induction of HSV-tk-specific immunity.[21,22] Several suicide mechanisms based on proteins of human origin may be less immunogenic than HSV-tk. A human caspase 9-based safety switch has recently been demonstrated to successfully halt an ongoing immune attack in a murine model for cell therapy-induced type 1 diabetes.[23,24] Our data support the use of human CD20 as non-immunogenic suicide mechanism. Several advantages of CD20 as safety switch can be envisioned: (i) CD20 has a dual function as selection marker and suicide gene, (ii) CD20+ cells are eliminated *in vivo* by RTX, which is widely used in the clinic, and (iii) RTX induces CDC, which is an extremely rapid form of cell death.

Recent studies in mice suggest that the formation of mixed TCR-dimers after *TCR* gene transfer may lead to severe autoimmunity (*G. Bendle and T. Schumacher, oral communication 26-28 May 2008*). We also observed off-target recognition by human T cells expressing mixed

TCR-dimers consisting of α and β chains exchanged between various naturally-occurring TCRs (*data not shown*). These findings illustrate the risk and capacity of mixed TCR-dimers to mediate autoimmunity and emphasize the relevance of co-transfer of a suicide mechanism in clinical TCR gene therapy. Our data show that CD20 can be successfully used as safety switch in TCR gene transfer and support the broad value of human CD20 as suicide gene in adoptive T-cell therapy.

## Authorship and Disclosures

MG, JHFF and MHMH designed research. MG, HMEvE and MGDK performed research. MG, HMEvE, JHFF and MHMH analyzed data. MG, RW, JHFF and MHMH wrote the manuscript.

The authors reported no potential conflict of interests.

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