

Antibody-dependent cellular cytotoxicity (ADCC) is mediated by genetically modified antigen-specific human T lymphocytes

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In the context of transplantation, donor and virus-specific T-lymphocyte infusions have demonstrated the dramatic potential of T cells as immune effectors. Unfortunately, most attempts to exploit the T-cell immune system against nonviral malignancies in the syngeneic setting have been disappointing. In contrast, treatments based on monoclonal antibodies (Abs) have been clinically successful and have demonstrated the clinical relevance of several antigens as therapeutic targets and the importance of the anti-

body-dependent cellular cytotoxicity (ADCC) pathway. In the present study, we considered the possibility of arming specific T cells with a receptor that would enable them to mediate ADCC. After transduction with a CD16/ γ receptor gene, CD4⁺ and CD8⁺ cytotoxic T lymphocytes displayed stable expression of the CD16 receptor at their surface. In the absence of Ab, CD16/ γ expression did not affect the capacity of specific T lymphocytes to kill their target following "natural" T-cell receptor recognition. When tested against

the autologous B-lymphoblastoid cell line (BLCL) coated with anti-CD20 mAb, the newly expressed Fc receptor enabled the T cells to kill the BLCL through ADCC. Adoptive transfer of such newly designed immune effector may be considered to increase antibody efficiency by harnessing the immune potential of T cells. (Blood. 2006;107:4669-4677)

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Introduction

The potential of transfused T lymphocytes as potent immune effectors is well documented. Such a potential was initially discovered in the allogeneic context from the clinical outcome of patients who received bone marrow transplants, where unselected donor T lymphocytes transfused together with hematopoietic precursors were found to be responsible for the graft-versus-host reaction as well as the graft-versus-leukemia effect.¹ Manipulation of this allogeneic effect through infusion of donor lymphocytes was found to induce durable remission in patients with chronic myelogenous leukemia and in patients with acute myelogenous leukemia relapse following allogeneic bone marrow transplantation.² Even more impressive was the restoration of viral immunity in immunodeficient patients after the transfer of cytomegalovirus (CMV)^{3,4} or Epstein-Barr virus (EBV)⁵⁻⁸-specific T lymphocytes. Unfortunately, attempts to harness the immune potential of T cells against nonviral malignancies apart from in the context of allogeneic transplantation have been largely unsuccessful, with only a few recent exceptions.⁹⁻¹² In contrast, with the limited positive experience using adoptive transfer of lymphocytes, the clinical benefit of antibody treatment is now well documented. Adoptive immunotherapy with mAbs targeting molecules such as CD20 or Her2/Neu recently has shown its capability to produce a clear clinical benefit,¹³ and it is thanks to these studies that the clinical pertinence of several antigens as immune therapeutic targets has been established. Such passively acquired antibodies can trigger apopto-

sis of tumor cells and activate complement-mediated (CDC) or antibody-dependent cellular cytotoxicity (ADCC) in treated patients. For rituximab, an anti-CD20 humanized mAb, several clinical observations suggested that ADCC mediated by Fc γ RIIIa (CD16)-bearing cells is a key mechanism of action. The gene coding Fc γ RIIIa displays a functional allelic dimorphism generating allotypes with either a phenylalanine (F) or a valine (V) residue at amino acid position 158. In vitro, natural killer (NK) cells from donors homozygous for Fc γ RIIIa-158V (VV) bound more human IgG1 and IgG3 than did NK cells from donors homozygous for Fc γ RIIIa-158F (FF).¹⁴ In vivo, Cartron et al recently have shown that the genotype homozygous for Fc γ RIIIa-158V (VV) is associated with a higher clinical response to rituximab in the treatment of follicular non-Hodgkin lymphomas (NHLs).¹⁵ For the anti-Her2/Neu humanized mAb trastuzumab, which is widely used to treat Her2/neu+ breast cancer, mechanisms thought to be responsible for the antitumor activity include down-modulation of the receptor, an antiangiogenic effect, complement-dependent cytotoxicity, a direct apoptotic effect, and ADCC. In fact, in a recent pilot study to elucidate the mechanism by which trastuzumab mediates its antitumor effect, Gennari et al observed that patients with complete or partial remission had a higher in situ leukocyte infiltration and a higher capacity to mediate in vitro ADCC.¹⁶ The findings of these clinical studies thus suggest that cancer patients eligible for mAb treatment are likely to benefit from efforts to optimize ADCC in vivo.

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Several effectors from both the innate and the adaptive immune system express CD16 receptors, including neutrophils, monocytes, a subset of NK cells, and rare T cells. Although each cell type is theoretically capable of ADCC, essentially all ADCC function in vitro was initially shown to be contained within a small fraction of lymphocytes expressing CD16.¹⁷⁻²¹ This also has been confirmed recently using newly developed therapeutic antibodies, in which case ADCC activity in vitro was shown to be mediated by NK cells but not by monocytes or neutrophils.^{22,23} Accordingly, NK cells would appear, at first glance, to be the most suitable cell type for an adoptive transfer protocol to improve a patient's ADCC potential. Along these lines, we have recently demonstrated the possibility of amplifying CD16⁺ NK cells from breast cancer patients eligible for trastuzumab treatment.²⁴ Although the amount of information concerning NK cells has increased at an impressive rate over the past decade, the lack of knowledge concerning the control of NK-cell proliferation, as well as their lifespan, potential for amplification, and in vivo recirculation, still severely limits their general use in the clinic. Such limitations are less pronounced with the use of T cells. In addition, more and more information concerning T-cell efficiency and fate after re-infusion into patients is becoming available. The expression of CD16 by a rare subset of T cells has been recognized for a long time,²⁵⁻³⁰ and the functional properties of such cells was first described by Lanier et al 20 years ago.³¹ The distinction between T $\gamma\delta$ and T $\alpha\beta$ T-cell subsets in their expression of CD16 was, however, made more recently. Rare observations also have been made of T-cell receptor (TCR) $\alpha\beta$ +CD16⁺ T cells in the peripheral blood of one particular healthy donor³² or from patients with granular lymphocyte proliferative disorders.³³ Within the TCR $\gamma\delta$ T-cell subset, the presence of CD16⁺ cells was first detected by Groh et al,³⁴ whereas the main CD16⁺ TCR $\gamma\delta$ T-cell subset was characterized only recently.^{35,36} CD16 expression by these lymphocyte subsets appears to be only transient³⁵ and is probably related to the acquisition of a terminal differentiation status.³⁶ Hence, despite T cells capable of ADCC being present in all individuals, they seem to represent rare and very specific subsets, thus rendering the manipulation of their ADCC function difficult to envisage.

Given (1) the potential in vivo efficiency of TCR $\alpha\beta$ T cells and the knowledge concerning their re-infusion, (2) the established pertinence of several antigens such as CD20 or Her2/neu as therapeutic targets, and (3) the likely influence of the ADCC pathway on the therapeutic efficiency of mAb treatments, we considered the possibility of improving the efficiency of mAb treatment by infusing effector T cells genetically modified to mediate ADCC. The first step toward achieving this goal and the aim of the present study was therefore to demonstrate that gene transfer can be used to design cytotoxic T cells with ADCC potential.

In human NK cells, Fc γ RIIIa (CD16) associates mainly with immunoreceptor tyrosine-based activation motif (ITAM)-containing homo-heterodimers of CD3 ζ and Fc ϵ RI γ .³⁷ Accordingly, an Fc γ RIIIa/Fc ϵ RI γ fusion protein was shown to elicit intracellular responses after transfection into the Jurkat cell line.^{38,39} In the present study, we constructed a chimeric molecule composed of the extracellular domain of Fc γ RIIIa (allotype V¹⁵⁸) and the transmembrane and cytoplasmic domain of Fc ϵ RI γ . This construct (referred to as CD16/ γ) was cloned into a lentiviral LNT-sffv vector,⁴⁰ or into a retroviral pMX vector,⁴¹ and used to transduce TCR $\alpha\beta$ T cells. To analyze TCR and CD16/ γ recognition by transduced T lymphocytes separately, we used T lymphocytes specific for allo- or viral antigens as target cells for CD16/ γ gene transfer. The

resulting CD16/ γ -transduced T cells retained their natural function (proliferation and cytotoxicity) through TCR recognition of cognate allo- or viral antigens and were able to kill Ab-coated autologous tumor target cells by ADCC mediated by the transduced chimeric CD16/ γ receptor.

Materials and methods

Cell lines

For lentiviral production we used a 293FT cell line (Invitrogen, Cergy Pontoise, France), a derivative of the 293F cell line that displays stable and constitutive expression of SV40 large T antigen under the control of the human CMV promoter. For retroviral production we used helper-virus-free Phoenix-Ampho packaging cells (GP, Nolan, Stanford, CA). 293FT and Phoenix cell lines were maintained in high-glucose (4.5 g/liter) Dulbecco modified Eagle medium (DMEM) (Sigma Aldrich, St Quentin Fallavier, France) supplemented with 10% fetal bovine serum (FBS) (Biowest, Nuaille, France) and 2 mM L-glutamine (Sigma Aldrich). Epstein-Barr virus B-lymphoblastoid cell lines (BLCLs) were derived from donor peripheral-blood mononuclear cells (PBMCs) by in vitro infection using EBV-containing culture supernatant from the Marmost B95.8 cell line from American Type Culture Collection (ATCC; Rockville, MD) in the presence of 1 μ g/mL cyclosporin-A. The Jurkat leukemia JRT3-T3.5 T-cell line (the β -negative variant of Jurkat that lacks TCR expression, from ATCC) was grown in RPMI 1640 culture medium (Sigma Aldrich) supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 UI/mL), and streptomycin (0.1 μ g/mL) (Biowest).

Construction of the Fc γ RIIIa/Fc ϵ RI γ chimeric gene encoding the CD16/ γ receptor

cDNA coding for the extracellular domain of CD16 (nucleotides 1 to 651, GenBank Accession No. X52645) was amplified by polymerase chain reaction (PCR) from a pcDNA3.1-Fc γ RIIIa (allotype V¹⁵⁸) plasmid kindly provided by Dr M. Ohresser and Dr H. Watier (EA 3853 Laboratoire d'Immunologie, Centre hospitalier Régional et Universitaire, Tours, France). cDNA for Fc ϵ RI γ (nucleotides 83 to 283, GenBank Accession No. BC033872) comprised a 2 amino-acid sequence (Pro4-Gln5) of the extracellular domain and the intact transmembrane and intracytoplasmic domains as previously described.³⁹ Fc ϵ RI γ cDNA was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using total RNA from cultured human NK cells and cloned in pcDNA3.1 (Invitrogen). Oligonucleotide primers (Sigma-Genosys, St Quentin Fallavier, France) used for the PCR reactions were as follows: CD16 sense: 5' GCG GGATCC TCT TTG GTG ACT TGT CCA 3'; CD16 antisense: 5' GCG GAA TTC CCC AGG TCG AAA GAA TGA 3'; gamma sense: 5' CCCTG GAATTC CCT CAG CTC TGC TAT ATC 3'; gamma antisense: 5' CATCTA GCGGCCCTCA CTG TGG TGG TTT C 3'. To generate the pcDNA3.1/Fc γ RIIIa/Fc ϵ RI γ , the 663-bp *Bam*HI-EcoRI Fc γ RIIIa fragment was ligated into the pcDNA3.1/Fc ϵ RI γ plasmid. The sequence of Fc γ RIIIa/Fc ϵ RI γ chimeric construct was verified (Genome Express, Meylan, France) and then cloned into a lentiviral LNT-sffv vector as well as into retroviral pMX vector.

Lentiviral vector production

LNT-sffv multicloning site (MCS) was kindly provided by Dr Howe (Molecular Immunology Unit, Institute of Child Health, London, United Kingdom).⁴⁰ VSV-G pseudotyped vectors were produced by transient transfection of 3 plasmids into 293FT cells using the ViralPower Lentiviral Expression system (Invitrogen).⁴² Three million 293FT cells were transfected by CaCl₂ precipitation with 12 μ g plasmid: 9 μ g ViralPower Packaging Mix (pLP1, pLP2, pLP/VSVG) and 3 μ g LNT-sffv/CD16-Fc ϵ RI γ . The medium (10 mL) was replaced 6 hours after transfection, and conditioned medium was collected 48 hours after transfection then filtered through 0.45- μ m pore-size filters. Viral particles were concentrated 100-fold by ultracentrifugation at 12 200 g for 90 minutes at 4°C. The viral

pellet was resuspended in phosphate-buffered saline (PBS) and kept at -80°C until use. Viral titer was determined by transduction of Jurkat T cells (1×10^5 cells per well in 96-well plates) with serial dilutions of virus and analyzed for CD16 expression at 3 to 5 days after infection. LNT-sffv/Fc γ RIIIa-Fc ϵ RI γ titers were typically 2 to 5×10^7 (infectious units) IU/mL.

Retroviral vector production

CD16 γ cDNA was cloned into *Bam*HI and NotI sites of the pMX vector.⁴¹ Transient retroviral supernatants were produced by transfection of Phoenix-Ampho packaging cells. Two million Phoenix-Ampho cells were seeded in 10-cm diameter dishes 24 hours prior to transfection. Transfection was performed with 6 μg pMX/CD16 γ plasmid DNA using FuGENE 6 reagent (Roche, Meylan, France). Conditioned medium was collected 48 hours after transfection, filtered through 0.45- μm pore-size filters, and kept at -80°C until use.

T-cell clone transduction using lentiviral supernatant

Clone 18-DO259 is a CD4⁺ cytolytic EBV (peptide 23 EBNA2)-specific human T-cell clone. Clones 4 and 31 are 2 CD4⁺ cytolytic HLA-DPB1*0401-specific human T-cell clones. Clone 31-DO.8 is a CD8⁺ cytotoxic CMV (peptide N9V/pp65)-specific T-cell clone. All clones were cultured using the following standard conditions: 1×10^6 T cells were stimulated in 96-well U-bottomed plates in the presence of irradiated (35 Gy) pooled allogeneic feeder cells (1×10^7 PBMCs and 1×10^6 BLCLs), 1 $\mu\text{g}/\text{mL}$ leucoagglutinin-A (Pharmacia, Uppsala, Sweden), and 300 UI/mL recombinant interleukin-2 (IL-2) (Roussel-Uclaff, Romainville, France). Five days after stimulation, T-cell clones were resuspended in RPMI 1640 culture medium (Sigma Aldrich) supplemented with 8% human serum and 300 UI/mL recombinant IL-2, seeded at 3×10^5 cells in 450 μL per well in 24-well plates, and exposed to lentiviral supernatant corresponding to a multiplicity of infection (moi) of 10 in the absence of polybrene. The culture medium was changed 24 hours after infection. Mock (nontransduced) controls were performed in parallel, but in this case no viral supernatant was added to the T-cell clones. Transduction efficiencies were assessed 5 days later.

Generation, expansion, and transduction of EBV-specific cytotoxic cell lines using retroviral supernatant

Donor PBMCs were plated in 24-well culture plates in RPMI 1640 culture medium (Sigma-Aldrich) supplemented with 8% pooled human serum (HS), 1% L-glutamine, 100 U/mL penicillin, and 0.1 $\mu\text{g}/\text{mL}$ streptomycin at 2×10^6 cells/well and stimulated with 5×10^4 35 Gray-irradiated autologous BLCL (PBMC/BLCL ratio of 40:1). After 10 days, T cells were collected and restimulated at a T/B ratio of 4:1 (5×10^5 T cells and 1.25×10^5 BLCL/well). IL-2 was added 3 days after the second stimulation. One day thereafter, retroviral transduction was performed by mixing 2×10^6 T cells (in 2 mL RPMI, 8% HS supplemented with 80 UI IL-2/mL) with 2 mL retroviral supernatant and 8 $\mu\text{g}/\text{mL}$ polybrene and then centrifuging at $2400 \times g$ for 90 minutes at 34°C . As a control, the T cells were incubated with nontransfected Phoenix-Ampho cell supernatant (nontransduced control cytotoxic T lymphocytes [CTL]). The day after transduction half of the medium was changed. Transduction efficiencies were assessed 3 days later and a third stimulation was performed 7 days after the second, in the presence of IL-2 and with an identical T/B ratio (4:1).

Immunoselection of transduced lymphocytes

Infected cells were analyzed by fluorescence-activated cell-sorter scanner (FACS) after staining with a mouse-antihuman CD16 mAb (3G8) and immunoselection using goat-antimouse-IgG1-coated beads (Dynabeads M-450, Dynal AS, Oslo, Norway) according to the supplier's instructions. Purity was greater than 95% according to CD16 expression.

Cytotoxicity assay

Cytotoxic activity was assessed using a standard ⁵¹Cr release assay. Target cells were labeled with 100 μCi (3.7 MBq) ⁵¹Cr for 1 hour at 37°C , washed 4 times with culture medium, and then plated at the indicated effector-to-target cell ratio in a 96-well flat-bottom plate. An autologous BLCL was used as a model of autologous tumor, and the humanized anti-CD20 mAb rituximab (Roche, Neuilly, France) was used (at 2 $\mu\text{g}/\text{mL}$) to induce ADCC. In some experiments, the anti-Her2/neu mAb trastuzumab (Roche) was used (at 10 $\mu\text{g}/\text{mL}$) as a control. For ADCC assays, the indicated monoclonal antibody was incubated with target cells for 20 minutes before addition of effector cells. In some experiments, autologous BLCLs were loaded with the HLA-A2 binding peptide NLVPMVATV (referred to as N9V) derived from the pp65 CMV phosphoprotein. For loading, target cells were incubated for 30 minutes at 37°C in the presence of different concentrations of peptides and were washed twice in RPMI-FBS. After a 4-hour incubation at 37°C , 25 μL of supernatant were removed from each well, mixed with 100 μL scintillation fluid, and ⁵¹Cr activity was counted in a scintillation counter. Each test was performed in triplicate. The results are expressed as the percentage of lysis, which is calculated according to the following equation:

$$(\text{experimental release} - \text{spontaneous release}) / (\text{maximal release}$$

$$- \text{spontaneous release}) \times 100$$

where experimental release represents the mean counts per minute (cpm) for the target cells in the presence of effector cells; spontaneous release represents the mean cpm for target cells incubated without effector cells; and maximal release represents the mean cpm for target cells incubated with 1% Triton X 100. For blocking experiments the F(ab')₂ fragment of the anti-human CD16-specific mAb 3G8 (Coger, Paris, France) was added at a concentration of 10 $\mu\text{g}/\text{mL}$ for the entire ADCC assay.

Cell line phenotyping

The following mAbs and their isotype controls were used: anti-CD16 (3G8)-PE or -PC5, anti-CD3-fluorescein isothiocyanate (FITC), anti-CD4-FITC, and anti-CD8-FITC (Beckman Coulter, Roissy, France). Two hundred thousand (0.2×10^6) cells were incubated for 10 minutes at room temperature in V-bottom microtiter plates in the presence of optimal concentrations of antibodies diluted with PBS supplemented with 5% HS in a final volume of 25 μL . After staining, plates were centrifuged, the supernatant was discarded by flicking, and wells were washed twice with 200 μL ice-cold PBS. Labeled cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

In vitro stimulation of T-cell clones

Stimulation of T-cell clones was performed in 96-well flat-bottom plates at 10^5 cells per well in 0.1 mL. In some experiments, 3300 BLCLs per well were used as target cells (effector-to-target ratio = 30:1) and humanized anti-CD20 (rituximab) (0.02 or 2 $\mu\text{g}/\text{mL}$) was used to induce ADCC. T-cell clones also were incubated with different concentrations of soluble rituximab (1 to 1000 $\mu\text{g}/\text{mL}$). As a positive control, T-cell clones were stimulated with 10 ng/mL phorbol myristate acetate (PMA) (Sigma) and 1 $\mu\text{g}/\text{mL}$ ionomycin (Sigma). Cells were cultured for 2 hours at 37°C in a humidified atmosphere of 5% CO₂ in air. Brefeldin-A was then added at 10 $\mu\text{g}/\text{mL}$, and the cells were cultured for an additional 4 hours at 37°C . Cells were transferred into 96-well V-bottomed plates, pelleted, resuspended in PBS, washed once more, and resuspended in PBS-2% formaldehyde (Euromedex, Nundolsheim, France). Cells were then fixed for 15 minutes at room temperature. Fixed cells were washed twice in PBS and stored in PBS at 4°C in the dark overnight.

Permeabilization and staining

Cells were pelleted and washed in 150 μL of $1 \times$ BD Phosflow Perm/Wash buffer (BD Biosciences Pharmingen, Le Pont de Claix, France) and resuspended in 50 μL $1 \times$ BD Phosflow Perm/Wash buffer for 20 minutes at room temperature. The following monoclonal antibodies (mAbs) were

used: PE-mouse anti-human tumor necrosis factor α (TNF α) (Mab1, BD Biosciences Pharmingen), PE-mouse anti-human interferon γ (INF γ) (B27, BD Biosciences Pharmingen) or with mouse IgG1 (BD Biosciences Pharmingen) as a negative control. Cells were stained at room temperature for 20 minutes with 50 μ L of the aforementioned PE-mAbs diluted 1:50 in 1 \times BD Phosflow Perm/Wash buffer. The cells were then pelleted, washed in 1 \times BD Phosflow Perm/Wash buffer followed by 2 further washes in PBS. For flow-cytometric analysis, data were collected and analyzed on a FACScan flow cytometer (BD Biosciences Pharmingen).

Proliferation assay

More than 3 weeks after the last stimulation, 2.5×10^4 resting T cells were cocultured (in triplicate) with 35 Gy-irradiated BLCL cells in 96-well flat-bottomed plates for 2 days at a responder-to-stimulator ratio of 1:1. Six hours before harvesting 1 μ Ci (0.037 MBq) of 3 H-thymidine was added to each well. 3 H-thymidine uptake was then measured in a liquid β scintillation counter (Betaplate, Wallac Oy, Finland). Results are expressed as mean value for each triplicate.

Results

Fc γ RIII/Fc ϵ RI γ vectors

cDNA encoding the chimeric CD16/ γ receptor, constructed as described in "Materials and methods," comprised the peptide signal and the extracellular domain (except the last 2 amino acids) of CD16, 2 amino acids of the extracellular domain, as well as the full transmembrane and the full intracytoplasmic domains of the Fc ϵ RI γ (Figure 1A). This construct was cloned into a lentiviral LNT-sfV vector⁴⁰ or into a retroviral pMX vector⁴¹ and viral titers determined on the Jurkat cell line. Persistence of CD16/ γ expression was evaluated on Jurkat cells after transduction using lentiviral supernatant, of which 98% were transduced after infection (Figure 1B). CD16/ γ expression was not detrimental to cell growth (data not shown), and after more than 3 months of culture, all cells still expressed high levels of CD16/ γ molecules (Figure 1B).

Generation of CD16/ γ T-cell clones

Four CD4⁺ and CD8⁺ antigen-specific T-cell clones were exposed for 24 hours to CD16/ γ lentiviral vector supernatant. After 5 days, clones were monitored for CD16/ γ expression by flow cytometry

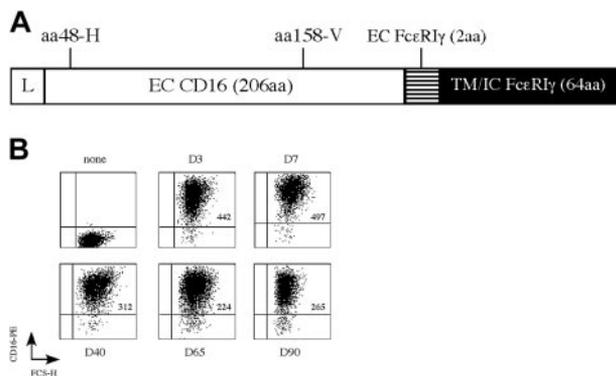


Figure 1. CD16/ γ vector design and expression in Jurkat cells. (A) Schematic representation of the chimeric Fc γ RIIIa-Fc ϵ RI γ molecule. The CD16/ γ chimeric cDNA comprised the leader (L) and the extracellular (EC) domain of CD16 (Fc γ RIIIa-158V allotype), 2 amino acids (aa) of the extracellular domain of the Fc ϵ RI γ , as well as the intact transmembrane (TM) and intracellular (IC) domains. (B) Maintenance of chimeric receptor expression in Jurkat cells transduced with 20 μ L of lentiviral virus stock. Transduced cells were analyzed by flow cytometry for CD16 expression over a 3-month period. Mean fluorescence intensities are indicated in each quadrant.

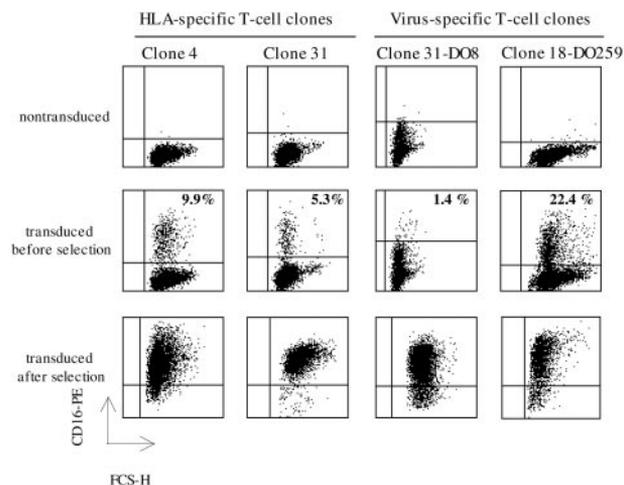


Figure 2. Generation of T-cell clones expressing CD16/ γ chimeric molecules. Five days after lentiviral transduction with the CD16/ γ gene (moi = 10:1), the T-cell clones were stained with CD16-PE (3G8) and analyzed by flow cytometry. Percentages of CD16/ γ -positive cells are indicated for each clone. Clones 4 and 31 are 2 cytolytic CD4⁺ HLA-DPB1*0401-specific T-cell clones, clone 31-DO8 is a CD8⁺ HLA-A*0201/CMV-pp65^{NSV}-specific T-cell clone, and clone 18-DO259 is a CD4⁺ cytolytic EBV-specific T-cell clone.

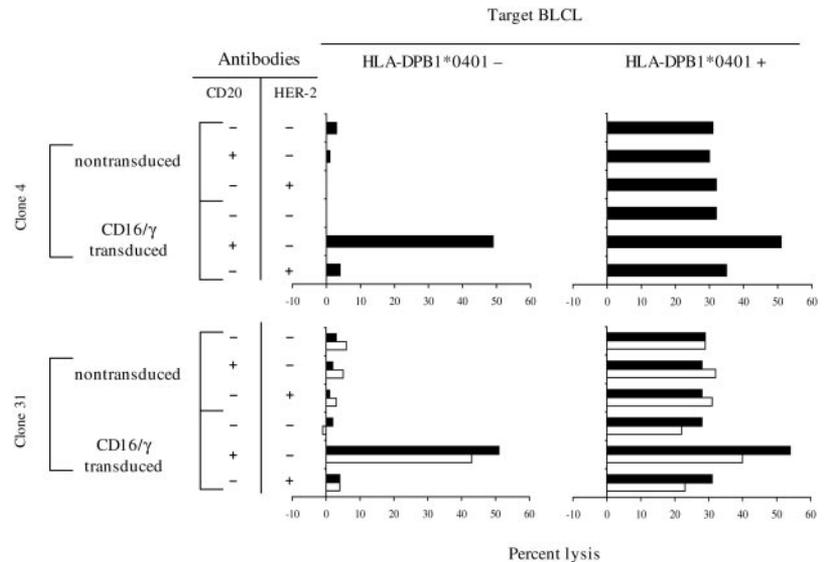
with a CD16-PE mAb. Transduction efficiencies ranged from 1.4% to 22.4% (Figure 2). After immunoselection using the 3G8 mAb, T-cell clones were further analyzed and shown to retain CD16/ γ expression at the same level during the entire follow-up period. In addition their CD3 expression remained identical to that observed in nontransduced T-cell clones (data not shown). Finally, the binding specificity of the human IgG isotypes for the T-cell clones was similar in our hands to that observed for purified NK cells (IgG3 > IgG1 > IgG2 > IgG4), and the binding was almost totally inhibited in the presence of saturating amounts of the anti-CD16 mAb 3G8 (data not shown).

ADCC by allospecific CD4⁺ T-cell clones expressing CD16/ γ chimeric molecules

Clone 4 and clone 31 are 2 allospecific HLA-DPB1*0401-specific T-cell clones. The ADCC activity of transduced and nontransduced clones was evaluated using a standard 4-hour 51 Cr release assay. Target BLCLs (all positive for CD20 and negative for Her2/neu antigens) that were either HLA-DPB1*0401 negative or positive were coated or not with the humanized anti-CD20 mAb rituximab or the humanized anti-Her2/neu mAb trastuzumab as a negative control before coculture with the T-cell clones.

Cytotoxic activity of clones 4 and 31 against the HLA-DPB1*0401-positive BLCL (the cognate target of their TCR) is shown on the right-hand panel of Figure 3. In the absence of mAb able to recognize the BLCL (no mAb or anti Her2/neu), the cytotoxic scores of transduced or nontransduced T-cell clones were identical, strongly suggesting that TCR recognition was unaffected by CD16/ γ transgene expression. In the presence of anti-CD20 mAb, only a slight increase in target-cell lysis was observed, reflecting the fact that for these T-cell clones, the cytotoxic activity was already almost maximal after TCR recognition. The cytotoxic scores against the HLA-DPB1*0401-negative BLCLs are shown on the left-hand panel of Figure 3. As expected, in the absence of mAb, the clones did not recognize the HLA-DPB1*0401-negative target cells. In contrast, both CD16/ γ transduced clones killed the HLA-DPB1*0401-negative BLCLs incubated with the anti-CD20 mAb. This cytotoxic activity was not observed in the presence of

Figure 3. TCR and CD16-mediated target-cell recognition by CD4⁺ HLA-DPB1*0401-specific cytolytic T-cell clones. Non-transduced and transduced T-cell clones were tested against ⁵¹Cr-labeled HLADPB1*0401-negative or -positive BLCLs. ADCC activities were assessed in presence of either rituximab (anti-CD20, 2 μg/mL) or herceptin (anti-HER-2, 10 μg/mL) as negative controls. Results are expressed as percentage of specific lysis (effector-to-target ratio = 30:1, mean of triplicate). For clone 31, ■ and □ represent 2 independent experiments.



the anti-Her2/neu mAb. Finally, cytotoxic activity by CD16/γ-transduced T-cell clones was found to be inhibited in the presence of anti-CD16 mAb F(ab')₂ fragments (Figure 4). Thus, cytotoxicity was dependent on CD16 membrane expression on the T-cell clones and on target-cell recognition by the mAb. Together, these data demonstrate that T-cell clones 4 and 31 had acquired the capacity to mediate ADCC after CD16/γ transduction. Interestingly, the cytotoxic activity of the transduced T-cell clones against the HLA-DPB1*0401-positive BLCLs and the HLA-DPB1*0401-negative BLCLs in the presence of anti-CD20 mAb was similar. Thus, the co-engagement of TCR and CD16 was not cooperative in T-cell clones. This observation is in line with a recent report showing that NKP46 engagement did not enhance CD16-dependent responses of NK cells⁴³ and supports the conclusion proposed by Bryceson et al that ITAM-based signals do not enhance one another.⁴³ Altogether, the results presented in Figures 3 and 4 demonstrate that CD16/γ transduction enabled T-cell clones to recognize Ab-coated target cells in the absence of TCR recognition and that TCR recognition was not affected by CD16/γ transgene expression.

CD16 crosslinking but not soluble mAb induced thymidine incorporation and cytokine production by CD16/γ-transduced T-cell clones

To test whether T-cell responses other than cytotoxic activity could be initiated in CD16/γ transduced T cells, several T-cell clones were tested for their ability to proliferate and produce cytokines (INFγ, TNFα, and IL-2) after CD16 exposure to antibody-coated cells. To exclude the possibility that soluble Ab can activate the clones, mAb concentrations of up to 1000 μg/mL were tested in the absence of target cells. Examples of results are presented in Figure 5: the specific proliferation (against the autologous BLCLs) of the CD8⁺ EBV-specific CD16/γ-transduced T-cell clone #24 was unaffected by the presence of mAb against CD20 or HER-2. In contrast, against the allogeneic BLCLs, the basic proliferation observed increased up to that observed against the specific target, in the presence of anti-CD20. This effect was not observed in the presence of anti-HER-2, suggesting that crosslinking was required to induce proliferation. Because the FcεRIγ signaling molecule was physically linked to the FcγRIIIa receptor, it was important to exclude the possibility that soluble Ab could stimulate the CD16/γ-transduced T cells. To this end, in the absence of BLCL, soluble

anti-CD20 was tested at concentrations of up to 1000 μg/mL. As shown in Figure 5A, no thymidine incorporation was detected at any concentration tested. The same conclusions could be drawn for cytokine production: the results obtained for TNF production by clone #7 are presented in Figure 5B. Essentially all cells from this clone were able to produce TNF when stimulated with PMA and Ca ionophore. Following crosslinking to target BLCLs, 22.5% of cells from the clone became positive for TNF. In contrast, in the absence of target cells, the soluble mAb was unable to induce significant TNF production by the clone, at concentrations of up to 1000 μg/mL. Three independent experiments were performed with 3 different CD16/γ-transduced T-cell clones and for 3 cytokines (TNFα, INFγ, and IL-2), leading to the same conclusion. The same results also were observed when testing human serum at a concentration of up to 50%.

TCR- and antibody-dependent recognition of the target cell by a CD16/γ transduced CD8⁺ HLA-A*0201/CMV-pp65^{N9V}-specific T-cell clone

To assess more precisely whether CD16/γ transduction could affect TCR signaling, we transduced a CD8⁺ HLA-A*0201/CMV-

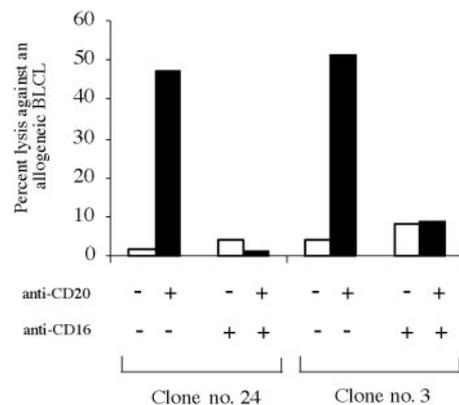


Figure 4. Anti-CD16 mAb blocks the target-cell recognition by CD16/γ-transduced T-cell clones. Effector cells (the CD8⁺ T-cell clone no.24 and the CD4⁺ T-cell clone no.3) were first incubated in the presence or absence of anti-CD16 mAb (3G8) F(ab')₂ fragments at 20 μg/mL. After 30' on ice effector cells were mixed (E/T ratio: 30:1) with an equal volume of ⁵¹Cr-labeled allogeneic EBV-LCL in the presence or absence of anti-CD20 mAb (rituximab, 0.2 μg/mL). Cytotoxicity was evaluated from ⁵¹Cr release after 4 hours of incubation; data represent mean from triplicate measurements.

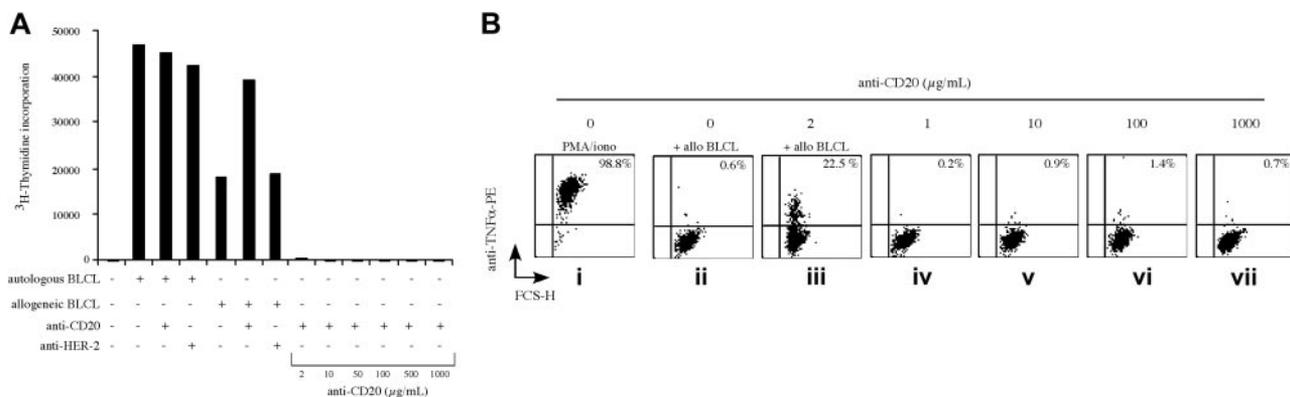


Figure 5. CD16/γ-transduced T-cell clone can proliferate and produce cytokines only when the CD16 molecule is crosslinked in the presence of mAbs and target cells. (A) Proliferative activity of CD16/γ-transduced EBV-specific T-cell clone no.24 was assessed after 72 hours of coculture with autologous or allogeneic BLCL and IL-2 (40 IU/mL) in the presence of either rituximab (anti-CD20, 2 μg/mL) or herceptin (anti-HER-2, 10 μg/mL). Soluble anti-CD20 mAb also was tested at the higher concentrations that are indicated. (B) The CD16/γ transduced EBV-specific T-cell clone no.7 (which recognizes through its TCR the autologous BLCL but not an allogeneic mismatch BLCL) and produced TNFα after PMA + ionomycin stimulation (i) was activated only after CD16 crosslinking in the presence of the allogeneic BLCL and 0.02 μg/mL of anti-CD20 (ii, iii) but remained unstimulated by the soluble mAb at a concentration of 50 to 50 000 superiors (iv-vii).

pp65^{N9V}-specific C31D08 T-cell clone. Nontransduced and transduced C31D08 clones were then tested against the autologous BLCLs loaded with varying concentrations of the HLA-A2 binding peptide NLVPMVATV (referred to as N9V) derived from the pp65 CMV phosphoprotein. According to the results shown on the top panel of Figure 6, BLCL lysis increased with increasing concentrations of N9V peptide, and maximal lysis was achieved at the same peptide concentration (50 nM) for both clones, strengthening the previous observation with allospecific T-cell clones that CD16/γ transgene expression did not affect TCR signaling. To assess ADCC activity, the autologous target BLCL was incubated with varying concentrations of the humanized anti-CD20 mAb. Confirming our previous results, in the absence of TCR signaling (ie, in the absence of the N9V peptide) the CD16/γ-transduced C31D08 cell clone was able to kill the BLCL in the presence of anti-CD20, according to a dose response that reached a plateau at 2 μg/mL.

Transduced EBV-specific CTLs expressed the CD16/γ transgene on both CD4⁺ and CD8⁺ T-cell subsets and showed increased cytotoxic activity against the autologous BLCLs in the presence of anti-CD20

An EBV-specific CTL line was generated from a seropositive healthy donor and transduced with a retroviral pMX-CD16/γ supernatant ("Materials and methods"). Flow cytometry analysis of CTLs stained with anti-CD16-specific antibody identified CD16

on 2.8% of the CTLs before transduction. These CD16⁺ lymphocytes were CD3⁻ and thus corresponded to the few NK cells present in the CTL population (Figure 7A). After transduction 14.0% of the CD3⁺ CTLs became CD16⁺ (Figure 7B). Notably, the level of CD16 expression on the CD3⁺ CTLs was very similar to that observed on the NK cells (Figure 7B). After immunoselection, staining of the transduced CTLs with CD16-PE and CD4⁻ or CD8-FITC mAb revealed the presence of 28.4% CD4⁺ and 67.5% CD8⁺ cells among the CD16⁺ lymphocytes (Figure 7C). These proportions were similar to those observed for nontransduced CTLs (20.4% and 77.9%, respectively, data not shown), showing that transduction was just as efficient for CD8⁺ cells as for CD4⁺ cells. Because of the presence of NK cells in the polyclonal population after CD16 purification, a panel of T-cell clones was derived from the CTLs, and examples of their ability to kill the autologous BLCLs in the presence or absence of anti-CD20 are shown in Figure 7D. For these CD8⁺ and CD4⁺ clones, which had a relatively low cytotoxic activity against the autologous BLCLs, a large increase in their ability to kill the target BLCL was observed when the BLCL was coated with anti-CD20. For these clones when both the TCR and CD16/γ chain molecule recognize the same target, the increase in cytotoxicity appeared different to that observed for the allospecific T-cell clones presented in Figure 3 ("Discussion"). The third CD8 clone in Figure 7C was presented as an example of a nonspecific T cell (often present in various proportions in such polyclonal cultures) that became an effector against the BLCL in the presence of anti-CD20. Hence, transduction of the CD16/γ chimeric receptor in polyclonal EBV-specific CTLs confers ADCC potential to both the CD4⁺ and CD8⁺ T-cell subsets.

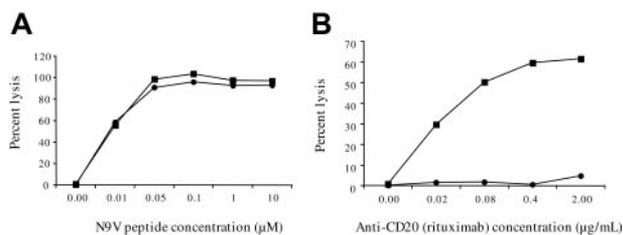


Figure 6. TCR and CD16 mediated target-cell recognition by HLA-A*0201/CMV-pp65^{N9V}-specific C31D08 T-cell clone. Nontransduced control and CD16/γ-transduced C31D08 T-cell clones were tested (A) against an HLA-A*0201-CD20⁺ autologous BLCL in the presence of the increasing concentrations of N9V peptide (to test TCR-dependent cytolytic activity) and (B) in the presence of a humanized anti-CD20 mAb (rituximab) (to assess ADCC activity). Both tests were performed in the same ⁵¹Cr-release assay. Results are expressed as percent of specific lysis (effector-to-target ratio = 30:1, mean of triplicate). In A and B, ● indicates nontransduced clone; ■, transduced clone.

Discussion

Our results demonstrate that human T cells can be modified by gene transfer to be conferred ADCC capacity. After transduction with a CD16/γ receptor gene, monoclonal and polyclonal CD4⁺ and CD8⁺ cytotoxic T lymphocytes displayed stable expression of the CD16 receptor at their surface. When tested against target BLCLs coated with the humanized anti-CD20 mAb rituximab, the newly expressed Fc receptor enabled the T cells to kill the BLCLs through ADCC. In the absence of mAb, the additional pathway of signaling

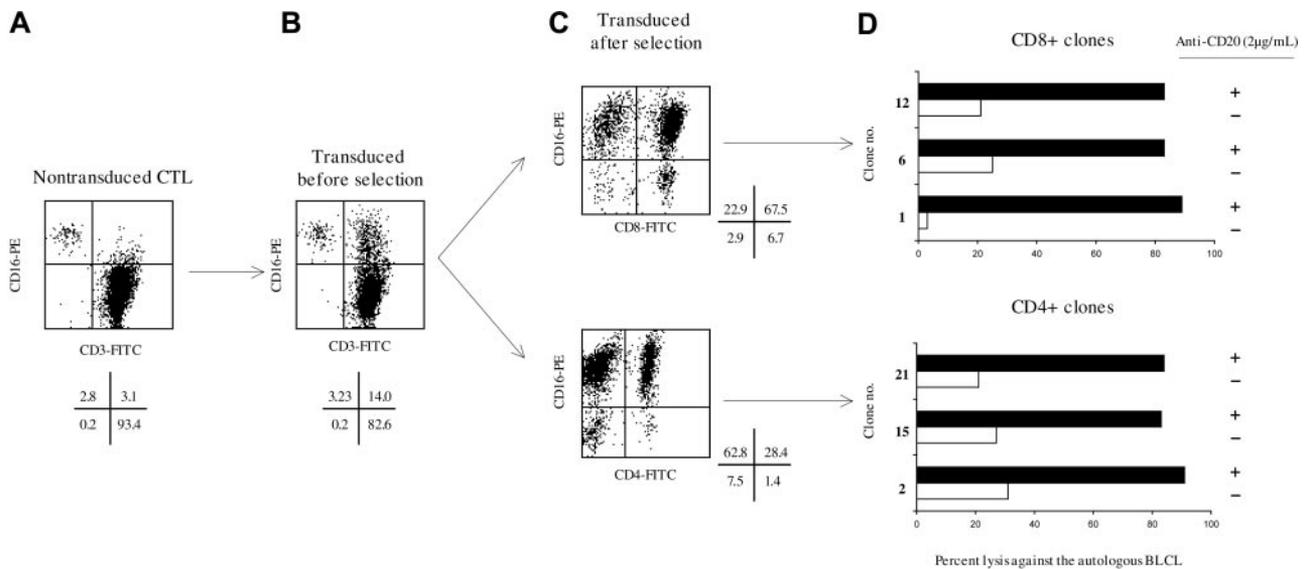


Figure 7. CD8⁺ and CD4⁺ polyclonal EBV-specific CTL express CD16/γ after transduction and mediate ADCC. EBV-specific CTLs were selected against the autologous BLCL and transduced with retroviral pMX-CD16/FcεRIγ according to the protocol described in "Materials and methods." Note in panel A that a few CD16⁺CD3⁻ cells (NK cells) were present among the CTL. After transduction 14% of the CTL expressed CD16, at a level comparable to that observed in NK cells still present in the culture (B). After immunoselection and restimulation, CTLs were stained with CD16-PE and CD4-FITC or CD8-FITC to analyze the proportion of transduced CD4 and CD8, respectively (C). Finally, a panel of CD4⁺ and CD8⁺ T-cell clones was derived from the CTL to precisely assess the effect of CD16/γ transduction on the cytolytic potential of CD4 and CD8 CTLs against autologous BLCL. In panel D, examples show the dramatic increase in cytolytic scores observed for both the CD4⁺ and CD8⁺ clones when tested against the autologous BLCL in the presence of anti-CD20 (effector-to-target ratio = 30:1). Note the example of clone CD8 no.1, which was probably not EBV specific but became an effector in the presence of mAb.

provided to the T cell did not affect its capacity to kill its target after "natural" TCR recognition.

Beyond the general interest of arming T cells for ADCC that is discussed below, the use of specific T cells to prove this concept led to the observation that even when T cells recognize their target cells through their TCR, in several cases they perform better in terms of cytotoxicity if they also are armed to mediate ADCC. Indeed, aside from the case where the target BLCL was loaded in vitro with high concentrations of CMV peptide, which does not correspond to a physiologic situation, when both the TCR and CD16/γ chain molecule recognize the same target, the increase in cytotoxicity appeared different for the allospecific (Figure 3) and the EBV-specific (Figure 7) T-cell clones. For the allospecific T cells, the cytotoxicity score was only slightly increased, while the increase was much more pronounced in the case of the EBV-specific T cells. Although further analysis will be necessary to describe the precise mechanism responsible for the increased recognition of the BLCL by the EBV-specific T cells in the presence of anti-CD20, a likely explanation is that the antigens recognized by the TCR of these clones are not presented by all cells of the BLCL. This could be the case for antigen encoded by immediate early and early lytic cycle proteins that are frequent targets of the EBV T-cell response.⁴⁴⁻⁴⁶ In such situations, only a fraction of the target cells express the antigen recognized by the TCR, while the whole population expresses the target for ADCC (CD20 in the present case). Moreover, for the allospecific T-cell clones, the difference was small and probably not significant, because all cells of the BLCL would be expected to present the alloantigen. Of note, other classic T-cell responses such as proliferation and cytokine production also are induced after CD16 crosslinking; and as is the case for TCR-mediated recognition, T-cell stimulation occurs only after crosslinking and not in the presence of soluble Ab.

The possibility of modifying any T cell so as to confer ADCC capacity upon it would make it possible to harness their well-known immune potential against antigens for which clinical

relevance has already been demonstrated by the recent clinical success of several therapeutic mAb. These results may have important implications for immunotherapy. In line with clinical applications, one could ask what the best-suited T-cell population for a CD16/γ gene transfer would be? The answer to this question may not be unique and will probably depend upon the clinical context. Candidate T lymphocytes for gene transfer can be easily obtained from peripheral-blood lymphocytes (PBLs).⁴⁷⁻⁵³ They also can be obtained from Ag-selected T cells,^{5,14,54} and in these latter cases, the T population can be clonally defined.^{55,56} Transducing total PBLs is apparently the simplest way to rapidly obtain CD16/γ effectors, since it only requires a step of nonspecific activation followed by a period of amplification to reach the number of cells sufficient for the injection(s). In this case, the major limitation associated with the use of unselected polyclonal T cells would be the poor definition of the final population. Using nonspecific stimulation, the vast majority of T cells will be able to proliferate and, most likely, many useless transduced T cells (ie, noncytotoxic effectors) will be re-injected. An alternative approach would be to purify and transduce an Ag-specific T-cell subset that already has proved efficient in vivo. This is the case for the EBV memory T-cell population. In fact, such a strategy already has been considered and proved feasible by Rossig et al,⁵⁷ who expressed antitumor chimeric TCR in Epstein-Barr virus-specific T lymphocytes. We also demonstrated in the present study that CD16/γ may be efficiently transfected into EBV-specific CTLs. As a consequence, the same gene transfer strategy becomes applicable in any clinical situation for which therapeutic efficiency relies at least in part on the patient's ADCC potential. Technically, the same gene provides the therapeutic function (CD16/γ) and the tag for specific T-cell selection (the extracellular portion of CD16). In this situation, following a procedure that we have recently described to select allospecific thymidine kinase-positive T lymphocytes,⁵⁶ we demonstrated that a single immunomagnetic selection, timely

performed after EBV-specific reactivation, allowed for the recovering of transduced T cells containing the therapeutic transgene (CD16/γ) and demonstrating the desired specificity (EBV).

A further step toward defining the transduced T cells would be to exhaustively characterize the composition of the T-cell population to be injected. Such an objective only can be achieved after cloning. In addition, in line with safety concerns, considering the use of clonal-cell populations would allow for their production using the procedure we described previously.⁵⁶ This procedure enables the recovery of T-cell clones with a chosen specificity and that are clonal also vis-a-vis the site(s) of transgene insertion. Indeed, preliminary analysis of several of the clones presented also suggests that they are clonal vis-a-vis the site of transgene insertion. From an immunologic point of view, the use of specific T cells whose TCR specificity is known should be safer than the use of a bulk population, because this is a definite way of preventing the transduction of T cells with unpredictable specificity. From a genetic point of view, this procedure is the only one that permits the use of a genetically homogeneous population of transduced T cells. Obviously, today, such a strategy does not appear realistic for a treatment that requires a cellular preparation for each patient, and for such applications PBLs or EBV-specific T cells appear to be best suited as targets for CD16/γ gene transfer. Nevertheless, although preparation of specific T cells on a patient-per-patient basis has proved feasible, such an approach always will be limited by several logistical problems and, in particular, the time delay

between the decision to include a patient and the production of a sufficient quantity of effector cells. Time constraints also will limit the level of characterization that can be achieved for the cells to be used. These difficulties have rendered the adoptive transfer of T cells poorly attractive for commercial development and, as a consequence, have limited its clinical applications. Finally, recent clinical reports using allogeneic T or NK lymphocytes have opened up the possibility of using allogeneic lymphocytes.⁵⁸⁻⁶¹ Provided that future trials using this strategy confirm the initial encouraging results, the use of allogeneic lymphocytes will extend rapidly. Obviously, in this setting, the absence of a potential alloreaction against the recipient will have to be formally documented before CTL infusion; this can be achieved using T-cell clones of documented specificities, such as those presented in this report. In conclusion, T-cell clones of documented specificity (directed against EBV or CMV, for example) and transduced with a chimeric CD16/γ transgene could probably become generic reagents to improve the clinical outcome of patients susceptible to benefit from an improved ADCC potential.

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