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# LEUKEMIA

## Normal and Malignant Hemopoiesis

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## The Journal of Normal and Malignant Hemopoiesis




**OFFICIAL JOURNAL OF THE LEUKAEMIA RESEARCH FUND U.K.**

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# Chimeric receptors with 4-1BB signaling capacity provoke potent cytotoxicity against acute lymphoblastic leukemia

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**To develop a therapy for drug-resistant B-lineage acute lymphoblastic leukemia (ALL), we transduced T lymphocytes with anti-CD19 chimeric receptors, consisting of an anti-CD19 single-chain variable domain (reactive with most ALL cases), the hinge and transmembrane domains of CD8α, and the signaling domain of CD3ζ. We compared the antileukemic activity mediated by a novel receptor ('anti-CD19-BB-ζ') containing the signaling domain of 4-1BB (CD137; a crucial molecule for T-cell antitumor activity) to that of a receptor lacking costimulatory molecules. Retroviral transduction produced efficient and durable receptor expression in human T cells. Lymphocytes expressing anti-CD19-BB-ζ receptors exerted powerful and specific cytotoxicity against ALL cells, which was superior to that of lymphocytes with receptors lacking 4-1BB. Anti-CD19-BB-ζ lymphocytes were remarkably effective in cocultures with bone marrow mesenchymal cells, and against leukemic cells from patients with drug-resistant ALL: as few as 1% anti-CD19-BB-ζ-transduced T cells eliminated most ALL cells within 5 days. These cells also expanded and produced interleukin-2 in response to ALL cells at much higher rates than those of lymphocytes expressing equivalent receptors lacking 4-1BB. We conclude that anti-CD19 chimeric receptors containing 4-1BB are a powerful new tool for T-cell therapy of B-lineage ALL and other CD19$^+$ B-lymphoid malignancies.**

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## Introduction

In approximately 20% of children and 65% of adults with acute lymphoblastic leukemia (ALL), drug-resistant leukemic cells survive intensive chemotherapy and cause disease recurrence.[1,2] For patients with recurrent disease or with certain adverse disease features, such as B-lineage ALL with the t(9;22)(q34;q11), hypodiploidy <45 chromosomes, or *MLL* gene rearrangements in infants, current chemotherapy regimens are mostly ineffective.[3] Significant improvements in cure rates require the development of treatments that bypass cellular mechanisms of drug resistance and that have high therapeutic indexes.

Clinical observations suggest that T lymphocytes can control the recurrence of chemotherapy-refractory leukemia. For example, T-cell-mediated graft-versus-host disease (GvHD) is associated with delay or suppression of leukemia relapse after allogeneic stem cell transplantation.[4–6] Infusions of donor lymphocytes can have antileukemic effects,[7–10] but they carry the risk of severe GvHD and their antileukemic effect is often inadequate in ALL.[8,11,12]

T-lymphocyte specificity can be redirected by the transduction of artificial immune receptors, which typically consist of an extracellular antibody-derived single-chain variable domain (scFv) and an intracellular signal transduction molecule (eg, CD3ζ).[13–15] Allogeneic or autologous T lymphocytes expressing these receptors can be activated by cell surface epitopes targeted by the scFv and kill the epitope-presenting cells. In ALL, CD19 is an attractive target because it is expressed on virtually all leukemic cells in around 85% of cases (ie, B-lineage ALL), it is not expressed by normal nonhematopoietic tissues, and among hematopoietic cells, it is only expressed by B-lineage lymphoid cells.[16–19] However, CD3ζ signaling may not be sufficient to produce a durable immune response; without a second signal, or costimulus, T cells rapidly undergo apoptosis after stimulation.[19–22] This is a central issue for T-cell therapy of ALL because ALL cells generally lack the ligands of CD28,[23] and of 4-1BB (C Imai, D Campana, unpublished observations), the two major T-cell costimulatory molecules.

In this study, we compared the function of human T cells expressing an anti-CD19-CD3ζ receptor to that of T cells expressing a novel chimeric receptor that contains the signal transduction domain of 4-1BB (CD137) as well as anti-CD19 scFv and CD3ζ (anti-CD19-BB-ζ). 4-1BB, a tumor necrosis factor-receptor family member, was selected because it prevents activation-induced death of T cells,[24–27] induces expansion of CD8$^+$ cells,[28] and enhances CD8$^+$ T-cell responses during viral infection and allograft rejection.[28–31] Most importantly, extensive experimental evidence with animal models of cancer points to a crucial role of 4-1BB signaling for effective antitumor responses.[32–36] We found that anti-CD19-BB-ζ-transduced T cells have powerful antileukemic activity: they can destroy CD19$^+$ ALL cell lines and primary leukemic cells at low effector: target (E:T) ratios and under conditions that approximate the *in vivo* microenvironment where leukemic cells grow.

## Materials and methods

### Cells

The human B-lineage ALL cell lines OP-1 [t(9;22)(q34;q11)/*BCR-ABL*],[37] and RS4;11 [t(4;11) (q21;q23)/*MLL-AF4*],[38] the T-cell lines Jurkat[39] and CEM-C7,[40] and the myeloid cell lines K562[41] and U-937[42] were available in our laboratory. Cells were maintained in RPMI-1640 (Gibco, Grand Island, NY, USA) with 10% fetal calf serum (FCS; BioWhittaker, Walkersville, MD, USA) and antibiotics. Human adenocarcinoma HeLa cells and embryonic kidney fibroblast 293T cells were maintained in

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Primary leukemia cells were obtained from patients with newly diagnosed B-lineage ALL with the approval of the St Jude Children's Research Hospital Institutional Review Board and with appropriate informed consent. The diagnosis of B-lineage ALL was unequivocal; in each case, more than 95% of leukemic cells were positive for CD19. Peripheral blood samples were obtained from healthy adult donors. Mononuclear cells were collected from the samples by centrifugation on a Lymphoprep density step (Nycomed, Oslo, Norway) and were washed two times in phosphate-buffered saline (PBS) and once in AIM-V medium (Gibco).

### *Plasmids*

The plasmid encoding anti-CD19 scFv was previously reported.[43] The pMSCV-IRES-GFP, pEQPAM3(-E), and pRDF were obtained from the St Jude Vector Development and Production Shared Resource. Signal peptide, hinge and transmembrane domain of CD8α, and intracellular domains of 4-1BB, CD3ζ, and CD19 were subcloned by PCR using a human spleen cDNA library (from Dr G Neale, St Jude Children's Research Hospital) as a template (Figure 1). We used splicing by overlapping extension by PCR (SOE-PCR) to assemble several genetic fragments.[44] The sequence of each genetic fragment was confirmed by direct sequencing. The expression cassettes were subcloned into *Eco*RI and *Xho*I sites of MSCV-IRES-GFP vector

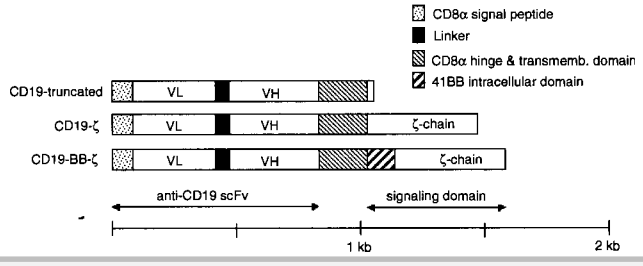
To transduce CD19-negative K562 cells with CD19, we constructed an MSCV-IRES-DsRed vector. The IRES and DsRed sequences were subcloned from MSCV-IRES-GFP and pDsRedN1 (Clontech, Palo Alto, CA, USA), respectively, and assembled by SOE-PCR. The IRES-DsRed cassette was digested and ligated into *Xho*I and *Not*I sites of MSCV-IRES-GFP. The expression cassette for CD19 was subsequently ligated into *Eco*RI and *Xho*I sites of MSCV-IRES-DsRed vector.

### *Virus production and gene transduction*

To generate RD114-pseudotyped retrovirus, we used calcium phosphate DNA precipitation to transfect $3 \times 10^6$ 293T cells, maintained in 10-cm tissue culture dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) for 24 h, with 8 μg of one of the vectors, anti-CD19-ζ, anti-CD19-BB-ζ, or anti-CD19-truncated, 8 μg of pEQ-PAM3(-E), and 4 μg of pRDF. After 24 h, the medium was replaced with RPMI-1640 with 10% FCS and antibiotics. Conditioned medium containing retrovirus was harvested 48 and 72 h after transfection, immediately frozen in dry ice, and stored at −80°C until use. HeLa cells were used to titrate virus concentration.

Peripheral blood mononuclear cells were incubated in a tissue culture dish for 2 h to remove adherent cells. Nonadherent cells were collected and prestimulated for 48 h with 7 μg/ml PHA-M (Sigma, St Louis, MO, USA) and 200 IU/ml human IL-2 (National Cancer Institute BRB Preclinical Repository, Rockville, MD, USA) in RPMI-1640 and 10% FCS. Cells were then transduced as follows. A 14-ml polypropylene centrifuge tube (Falcon) was coated with 0.5 ml of human fibronectin (Sigma) diluted to 100 μg/ml for 2 h at room temperature and then incubated with 2% bovine serum albumin (Sigma) for 30 min. Prestimulated cells ($2 \times 10^5$) were resuspended in the fibronectin-coated tube in 2–3 ml of virus-conditioned medium with polybrene (4 μg/ml; Sigma) and centrifuged at 2400 *g* for 2 h. The multiplicity of infection (4–8) was identical in each experiment comparing the activity of different chimeric receptors. After centrifugation, cells were left undisturbed for 24 h in a humidified incubator at 37°C, 5% $CO_2$. The transduction procedure was repeated on two successive days. Cells were then washed twice with RPMI-1640 and maintained in RPMI-1640, 10% FCS, and 200 IU/ml of IL-2 until use.

A similar procedure was used to express chimeric receptors in Jurkat cells, except that cells were not prestimulated. K562 cells expressing CD19 were created by resuspending $2 \times 10^5$ K562 cells in 3 ml of MSCV-CD19-IRES-DsRed virus medium with 4 μg/ml polybrene in a fibronectin-coated tube; the tube was centrifuged at 2400 *g* for 2 h and left undisturbed in an incubator for 24 h. Control cells were transduced with the vector only. These procedures were repeated on 3 successive days. After confirming CD19 and DsRed expression, cells were subjected to single-cell sorting with a fluorescence-activated cell sorter (MoFlo, Cytomation, Fort Collins, CO, USA). The clones that showed the highest expression of DsRed and CD19 and of DsRed alone were selected for further experiments.




### *Detection of chimeric receptor expression*

Cells were stained with goat anti-mouse $(Fab)_2$ polyclonal antibody conjugated with biotin (Jackson Immunoresearch, West Grove, PA, USA) followed by streptavidin conjugated to peridinin chlorophyll protein (PerCP; Becton Dickinson, San Jose, CA, USA). Anti-CD4 and anti-CD28 antibodies conjugated to PE and anti-CD8 conjugated to PerCP (from Becton Dickinson, and Pharmingen, San Diego, CA, USA) were also used. Antibody staining was detected with a FACScan flow cytometer (Becton Dickinson).

For Western blotting, $2 \times 10^7$ cells were lysed in 1 ml RIPA buffer (PBS, 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% SDS) containing 3 μg/ml of pepstatin, 3 μg/ml of leupeptin, 1 mM of PMSF, 2 mM of EDTA, and 5 μg/ml of aprotinin. Cell lysates were separated by SDS-PAGE on a 12% acrylamide gel (BioRad, Hercules, CA, USA). After transfer to a PVDF membrane, this was incubated with a mouse anti-human CD3ζ (clone 8D3; Pharmingen) and then with a goat anti-mouse IgG horseradish peroxidase-conjugated antibody. Antibody binding was revealed by using the ECL kit (Pharmacia, Piscataway, NJ, USA).

### *Expansion of receptor-transduced primary T cells and IL-2 production*

Receptor-transduced lymphocytes ($3 \times 10^5$) were cocultured with $1.5 \times 10^5$ irradiated OP-1 cells in RPMI-1640 with 10% FCS with or without exogenous IL-2. Cells were pulsed weekly with irradiated target cells at an *E:T* ratio of 2:1. Viable cells

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