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## Chapter 7.

IL-7 receptor expression identifies suicide gene-modified allospecific CD8<sup>+</sup> T cells capable of self-renewal and differentiation into antileukemia effectors



# **IL-7 receptor expression identifies suicide gene-modified allospecific CD8<sup>+</sup> T cells capable of self-renewal and differentiation into antileukemia effectors**

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**In allogeneic hematopoietic cell transplantation (HSCT), donor T lymphocytes mediate the graft-versus-leukemia (GvL) effect, but induce graft-versus-host disease (GvHD). Suicide gene therapy, i.e. the genetic induction of a conditional suicide phenotype into donor T cells, allows dissociating the GvL effect from GvHD. Genetic modification with retroviral vectors after CD3 activation reduces T-cell alloreactivity. We recently found that alloreactivity is maintained when CD28 costimulation, IL-7 and IL-15 are added. Herein, we used the minor histocompatibility (mH) antigens HA-1 and H-Y as model alloantigens to directly explore the antileukemia efficacy of human T cells modified with the prototypic suicide gene Herpes simplex virus thymidine kinase (tk) after activation with different stimuli. Only in the case of CD28 costimulation, IL-7 and IL-15, the repertoire of tk<sup>+</sup> T cells contained HA-1- and H-Y-specific CD8<sup>+</sup> cytotoxic T-cells (CTL) precursors. Thymidine kinase-positive HA-1 and H-Y-specific CTLs were capable of self-renewal and differentiation into potent antileukemia effectors in vitro, and in vivo in a humanized mouse model. Self-renewal and differentiation coincided with IL-7 receptor expression. These results pave the way to the clinical investigation of T cells modified with a suicide gene after CD28 costimulation, IL-7 and IL-15 for a safe and effective GvL effect.**

## **INTRODUCTION**

Adoptive T-cell therapy of cancer, i.e. the transfer of tumor-reactive T lymphocytes for cancer treatment, is curative in the case of virus-associated lymphoproliferative disorders<sup>1</sup> and can induce long-term remission in patients with metastatic melanoma<sup>2,3</sup>. Allogeneic hematopoietic stem cell transplantation (HSCT) is a form of adoptive T-cell therapy of cancer<sup>4</sup>, in which donor alloreactive T cells play a crucial therapeutic graft-versus-leukemia (GvL) and a putative graft-versus-tumor effect<sup>5</sup>. The widespread exploitation of HSCT for cancer treatment is however limited by the frequent occurrence of graft-versus-host disease (GvHD), caused by the attack of donor alloreactive T cells against the recipient's healthy tissues. The implementation of a suicide gene in donor T lymphocytes is a promising strategy for the operational dissociation of the GvL effect from GvHD<sup>6</sup>. A suicide gene encodes for a factor that confers sensitivity to a non-toxic prodrug, enabling the selective elimination of expressing cells upon administration of the compound. In patients treated with donor T cells modified *ex vivo* with a retroviral vector (RV) to express the Herpes simplex-virus thymidine kinase (*tk*) suicide gene, the time-wise administration of the prodrug ganciclovir (GCV) blocked GvHD progression both in the HLA-identical<sup>7</sup> and in the HLA-mismatched HSCT setting<sup>8</sup>, while sparing a significant GvL effect.

Despite these encouraging results, the overall efficiency of suicide gene therapy in HSCT is limited because gene-modified human T lymphocytes produced with current clinical protocols are less alloreactive than unmodified cells<sup>9</sup>. This is confirmed by the observation that patients treated with *tk*<sup>+</sup> donor T cells experience less GvHD than expected. Given the close clinical of GvHD with the GvL effect, it is postulated that suicide gene therapy would greatly benefit from the preservation of T-cell alloreactivity. Others and we showed that the phenomenon of reduced alloreactivity of gene-modified T cells is not due to the integration of the transgene itself, but rather to the overall effect of the different steps of *in vitro* T-cell manipulation required for genetic modification with RV<sup>10,11</sup>. Genetic modification of T cells with RV requires proliferation, which is classically induced by the activation with anti-CD3 monoclonal antibodies (mAb) and culturing with high-dose IL-2, and results in T-cell differentiation<sup>12</sup>. There is growing evidence that alloreactivity may segregate at certain stages of T-cell differentiation. Upon antigen encounter, naïve T lymphocytes (T<sub>NA</sub>) proliferate and differentiate into effector and memory cells<sup>13</sup>. The pool of human memory T cells is functionally heterogeneous. The expression of a lymphnode addressin, such as CCR7 or CD62L, identifies central memory T cells (T<sub>CM</sub>), a subset of CD45RA<sup>-</sup> cells with high proliferative potential, but with poor effector function. Conversely, effector memory T cells (T<sub>EM</sub>), identified as CD45RA<sup>+</sup>/CCR7<sup>-</sup> or CD62L<sup>-</sup> cells, are cells with immediate effector function but with limited proliferative potential. Signal strength possibly controls the transition of T<sub>CM</sub> to T<sub>EM</sub> cells, thus ensuring the plasticity of the adaptive immune response *in vivo*<sup>14</sup>. Earlier studies in mouse models showed that, as opposed to T<sub>NA</sub> cells, the adoptive transfer of CD4<sup>+</sup> memory T cells did not cause GvHD<sup>15,16</sup>. Later, it was demonstrated that the adoptive transfer of a subset of CD8<sup>+</sup> memory T cells, possibly coinciding with human T<sub>CM</sub> cells, can both induce GvHD and mediate the GvL effect<sup>17</sup>. RV-mediated transduction after CD3 activation and culturing with high-dose IL-2 enriches for T<sub>EM</sub> cells. CD28 costimulation through anti-CD3 and anti-CD28 mAb coupled to cell-sized paramagnetic beads (CD3/CD28-beads) decreases the need for exogenous IL-2 and leads to gene-modified T<sub>CM</sub> cells<sup>12</sup>. In a xenograft model of GvHD using NOD/scid mice, *tk*<sup>+</sup> human T cells generated after CD28 costimulation with beads and culturing with low-dose IL-2 were more potent than those generated after CD3 activation and culturing with high-dose IL-2, but less than unmodified T cells<sup>12</sup>. In a fully humanized model of GvHD based on the grafting of HLA-mismatched skin onto NOD/scid mice, we recently showed that *tk*<sup>+</sup> T cells generated after CD28 costimulation with beads and culturing with a combination of IL-7 and IL-15 are as alloreactive as unmodified cells<sup>18</sup>. In the HLA-matched setting, which comprises the majority of HSCT, GvHD and the GvL effect are mediated by donor T cells directed against minor histocompatibility (mH) antigens present in the host but absent in the donor<sup>19</sup>. Minor H antigens are allopeptides derived from polymorphic intracellular proteins presented and recognized in the context of HLA. In HLA-matched, mH antigen-mismatched HSCT, the association between leukemia clearance and *in vivo* emergence of high-avidity cytotoxic T lymphocytes (CTLs) specific for the mH antigens HA-1 and HA-2 suggests a key role for mH antigens in the GvL effect<sup>20</sup>. Moreover, we recently demonstrated that targeting HA-1 with CTLs is effective in controlling human leukemia<sup>21</sup> and solid tumors<sup>22</sup> in NOD/scid mice.

All the studies reported so far anticipated the GvL potential of suicide gene-modified human T lymphocytes using GvHD as a surrogate marker. In the current study, we aimed at directly exploring the antileukemia efficacy of T cells modified with the prototypic suicide gene *tk* using the mH antigens HA-1 and H-Y as model alloantigens.

## **MATERIALS AND METHODS**

### **Genetic modification of human T lymphocytes**

After informed consent, buffy coats or leukoapheresis products were obtained from HLA-A2<sup>pos</sup> healthy blood donors negative for the mH antigen HA-1 (n=3, HA1-*tk* #1, HA1-*tk* #2 and HA1-*tk* #3) or for the mH antigen H-Y (n=4, HY-*tk* #1, HY-*tk* #2, HY-*tk* #3, HY-*tk* #4). HA-1 negativity was defined as homozygosity for the non-immunogenic R isoform by molecular typing (R/R). PBMC were isolated by density-gradient centrifugation (Fresenius, Oslo, Norway), seeded in 6-well plates at  $1 \times 10^6$  cells/ml in IMDM (GIBCO-BRL, Gaithersburg, MD) supplemented with antibiotics, glutamine (GIBCO-BRL) and 10% heat-inactivated fetal calf serum (FCS, BioWhittaker, Milano, Italy), and activated with anti-CD3 mAb (OKT3, 30 ng/mL, Ortho Biotech, Raritan, NJ) or with CD3/CD28-beads (ClinExVivo CD3/CD28, Invitrogen, Carlsbad, CA) at a 3:1 bead/T-cell ratio. After CD3 activation, T cells were cultured with recombinant human IL-2 at 600 IU/mL (Chiron, Emeryville, CA). After CD3/CD28-bead activation, T cells were cultured with either IL-2 at 200 IU/mL, with IL-7 at 5 ng/mL (Peprotech, London, UK) or with a combination of IL-7 and IL-15 at 5 ng/mL (Peprotech, London, UK). At day two and three, T cells were resuspended in the SFCMM-3<sup>23</sup> or in the Mut2 RV supernatant (Molmed SpA, Milan, Italy) and centrifuged at 2400 rpm for 2 hours at 32°C (spinoculation). The Mut2 RV has been modified to avoid alternative splicing leading to loss of function<sup>24</sup>. After spinoculation, CD3/CD28-beads were removed and T cells cultured for additional 7-10 days in the presence of cytokines.

### **Flow cytometry**

Transduction efficiency, memory T-cell phenotype, intracellular cytokine production, enumeration and sorting of antigen-specific T cells with HLA-A2/peptide tetramers, annexin V/PI binding and human chimerism in mouse blood were evaluated by flow cytometry. The following monoclonal antibodies were used: fluorescein iso-thio-cyanate (FITC)-conjugated anti-human CD45RA, CD27 and IFN- $\gamma$ , phycoerythrin (PE)-conjugated anti-human CD8, nerve growth factor receptor (NGFR), CCR7, CD28, CD127 (IL-7R $\alpha$ ) and IL-2, peridinin chlorophyll-a protein (PerCP)-conjugated anti-mouse CD45 (Ly5.1), and allophycocyanin (APC)-conjugated mAbs to human CD3 and CD8. All mAbs were purchased from BD Biosciences (San Jose, CA) and FITC-conjugated annexin V from Bender MedSystems (Wien, AUT). APC or PE-conjugated HLA-A2 tetramers were produced in house and bound to the following peptides: human HA-1 allelic isoform H (VLHDDLLEA), human H-Y (FIDSYICQV), CMV pp65 (NLVPMVATV), EBV BMLF-1 (GLCTLSVAML). All samples were run on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) and analyzed with the CellQuest software (Becton Dickinson).

### **Generation and expansion of human mH antigen-specific CTLs**

DCs were obtained by immunomagnetic bead selection (CD14 microbeads, Miltenyi Biotec, Utrecht, NL) and culture of monocytes in RPMI medium (GIBCO-BRL), supplemented with 10% FCS, human recombinant granulocyte-monocyte colony-stimulating factor (GM-CSF, Peprotech) at 800 IU/ml and IL-4 (Peprotech) at 1  $\mu$ g/ml. After 5 days, lypopolisaccharide (Sigma-Aldrich, Zwijndrecht, NL) was added at 1  $\mu$ g/ml for 18 hours. At day 6, DCs were pulsed with either the human HA-1 allelic isoform H (VLHDDLLEA), the human H-Y (FIDSYICQV), the CMV pp65 (NLVPMVATV) or the EBV BMLF-1 (GLCTLSVAML) peptide at and co-cultured with T cells at 10:1 T-cell/DC ratio. After sorting by flow

cytometry, mH antigen-specific CTLs were cultured with irradiated autologous PBMC (30 Gy), PHA (GIBCO-BRL) at 2 ng/mL and IL-2 at 100 IU/mL.

### **Functional characterization of human mH antigen-specific CTLs**

*In vitro* cytotoxicity was evaluated in a standard  $^{51}\text{Cr}$  release assay described elsewhere<sup>21</sup>. Specific lysis was calculated as follows: (experimental release - spontaneous release) / (maximal release - spontaneous release) x 100. HLA-A2<sup>pos</sup> lymphoblastoid cell lines (LCL) or HLA-A2<sup>pos</sup> leukemic cells were used as targets. Leukemic cells were either derived from patients after informed consent or from the ALL-CM leukemic cell line. HA-1 positivity was defined as heterozygosity (H/R) or homozygosity (H/H) for the immunogenic H isoform. LCL or leukemic cells of male origin were assumed to be H-Y<sup>pos</sup>. ALL-CM is HLA-A2<sup>pos</sup>/HA-1<sup>pos</sup>/H-Y<sup>pos</sup> cell line is derived from a chronic myelogenous leukemia patient suffering from a Philadelphia chromosome-positive lymphoid blast. A HLA-A2-restricted HA-1-specific CTL clone (1.7) and a HLA-A2-restricted H-Y-specific CTL clone (21-17) were used as positive controls and, in some cases, as reference. When used as reference, the cytotoxicity of mH antigen-specific CTLs was calculated by dividing the specific lysis of the mH antigen-specific CTLs against the relevant mH antigen-expressing LCL with that of the respective mH antigen-specific CTL clone at the same 20:1 ratio. Minor Hag-independent survival was evaluated by plating mH antigen-specific CTLs at  $1 \times 10^6$ /mL in complete IMDM medium supplemented or not with IL-7 at 5 ng/mL. At different time-points, apoptotic cells were enumerated by flow cytometry after annexin V/PI staining. Mean telomere length was determined by quantitative *in situ* hybridization with flow-cytometry (flow FISH) using a FITC-conjugated telomere probe (DAKO, Copenhagen DK) and the 1301 subline of the human T lymphoblastic leukemia cell line CEM as a telomere length control. Relative telomere length was derived after normalization for each donor as follows: mean telomere length of population of interest / mean telomere length of PBL from the same donor. Minor Hag-specific cytokine production was evaluated by intracytoplasmic staining after coincubation of mH antigen-specific CTLs with the relevant mH antigen-expressing cells at 1:1 ratio for 6 hours at 37°C. The functionality of the *tk* suicide gene machinery was evaluated by measuring the  $^3\text{H}$ thymidine incorporation of mH antigen-specific CTLs in response to autologous feeder and PHA at increasing concentrations of the prodrug GCV. Inhibition of proliferation was calculated as follows: 1 - (incorporation in the presence of GCV / incorporation in the absence of GCV) x100.

### **In vivo evaluation of the antileukemia efficacy of human mH antigen-specific CTLs**

Antileukemia efficacy was evaluated in a humanized model for adoptive T-cell therapy described elsewhere<sup>21</sup>. Briefly, after approval from the Leiden University Medical Center review board on animal studies, 6 to 8 week-old NOD/scid mice female were treated with a rat anti-mouse IL-2/IL-15R $\beta$  mAb (1 mg/mouse i.p., TM $\square$ -1, kindly provided by Prof. Tanaka, Tokio, Japan). The day after,  $10^7$  ALL-CM cells were inoculated via the tail vein. After three days,  $2 \times 10^7$  antigen-specific CTLs were infused through the same route. During the first week from infusion, mice were given rhIL-2 i.p. ( $2 \times 10^4$  IU/mouse daily). ALL-CM chimerism was monitored by weekly bleeding through the tail vein. After osmotic lysis, flow cytometry was used to measure the relative percentage of human cells over total nucleated cells. CTL chimerism was calculated as follows: human CD3<sup>+</sup> cells / (human CD3<sup>+</sup> + mouse CD45<sup>+</sup> cells) x100. ALL-CM chimerism was calculated as follows: human CD45<sup>+</sup>/CD3<sup>-</sup> cells / (human



CD45<sup>+</sup>/CD3<sup>-</sup> + mouse CD45<sup>+</sup> cells) x log100. When ALL-CM chimerism was >70% mice had to be sacrificed for ethical reasons.

### **Statistical analysis**

Paired data were analyzed with a two-tailed Student t test, unpaired data with a two-tailed Mann-Whitney u test and two-variable data with linear regression. Leukemia-free survival in NOD/scid mice was analyzed with a generalized model based on linear regression (Prism, GraphPad, CA).

## RESULTS

### RV-mediated tk transduction after CD28 costimulation and culturing with IL-7/IL-15 preserves human mH antigen-specific CTL alloreactivity in vitro

In vitro stimulation of T lymphocytes from HLA-A2<sup>pos</sup>/HA-1<sup>neg</sup> healthy donors with autologous DC loaded with HLA-A2-restricted HA-1 peptides and IL-2 induces substantial numbers of HA-1-specific CTLs that can be isolated with HLA-A2<sup>HA-1</sup> tetramers<sup>25</sup>. This protocol was used to identify the requirements for the preservation of gene-modified HA-1-specific CTL alloreactivity. Hereto, after RV-mediated tk transduction in different conditions, T cells from HLA-A2<sup>pos</sup>/HA-1<sup>neg</sup> donors were stimulated with HA-1 peptide-loaded DC. Unmodified T cells from the same individuals were stimulated in parallel as control. The different conditions for transduction included the activation with an anti-CD3 mAb and culturing with high dose IL-2 (OKT3), according to our current clinical protocol<sup>8</sup>, or the activation with CD3/CD28-beads and culturing with either low-dose IL-2 (B+IL-2), with IL-7 (B+IL-7) or with a combination of IL-7 and IL-15 (B+IL-7/IL-15). IL-15 alone was not used because it promotes the differentiation into T<sub>EM</sub> cells<sup>26</sup>. A clinical-grade RV vector, which encodes for the tk suicide gene along with a truncated form of NGFR, was used in all conditions. As expected, transduction efficiency, final tk<sup>+</sup> T-cell yields and CD4/CD8 were higher in the case of previous activation with CD3/CD28-beads<sup>18</sup>, regardless of the cytokine used for culturing, than with OKT3 (Table I).

**Table I.** RV-mediated tk transduction and induction of human mH antigen-specific CTLs

<sup>a</sup> Condition	<sup>b</sup> Trans. (%)	<sup>c</sup> Yields (x10 <sup>6</sup> )	<sup>d</sup> CD4/CD8 (n)	<sup>e</sup> HLA-A2 <sup>HA-1</sup> tet <sup>+</sup> (%)	<sup>e</sup> HLA-A2 <sup>H-Y</sup> tet <sup>+</sup> (%)
<b>PBL</b>	-	10	2,1±0,3	2,4 (2,1-3,5)	2,7 (1,4-3,7)
<b>OKT3</b>	18,4±4,6	23±6,3*	1,1±0,2*	0,7 (0,4-1,2)	0,5 (0,2-0,9)
<b>B+IL-2</b>	41,1±8,1*	65±11,3*	1,8±0,2	1,0 (0,4-1,4)	0,8 (0,3-1,2)
<b>B+IL-7</b>	36,7±7,5*	47,3±6,4*	2,2±0,2	0,3 (0,1-0,5)	0,7 (0,5-0,9)
<b>B+IL-7/IL-15</b>	40,1±9,6*	70,1±11,3*	1,8±0,2	3,1 (2,8-4,1)	2,4 (2,1-4,7)

<sup>a</sup> Conditions for transduction: OKT3, anti-CD3 mAb and IL-2, B+IL-2, CD3/CD28-beads and IL-2, B+IL-7, CD3/CD28-beads and IL-7, B+IL-7/IL-15, CD3/CD28-beads, IL-7 and IL-15

<sup>b</sup> Transduction efficiency

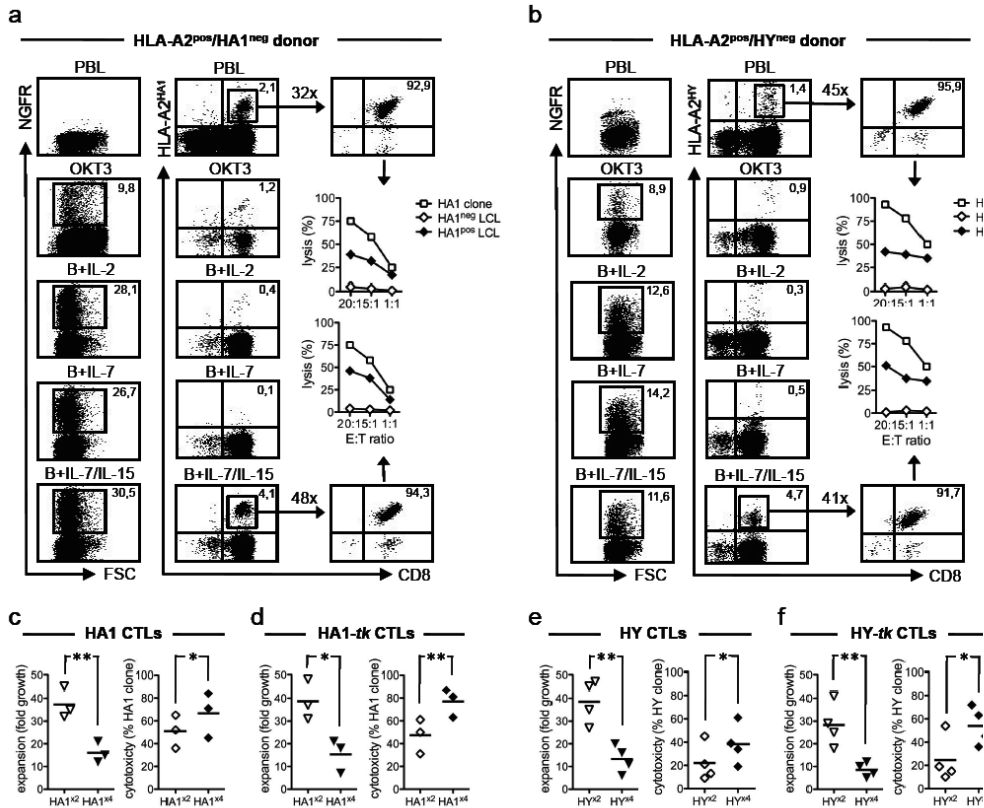
<sup>c</sup> NGFR<sup>+</sup> T-cell yields after transduction

<sup>d</sup> CD4/CD8 ratio on NGFR<sup>+</sup> cells

<sup>e</sup> HLA-A2<sup>HA-1</sup> or HLA-A2<sup>H-Y</sup> tetramer binding on NGFR<sup>+</sup>/CD8<sup>+</sup> T cells

In all donors tested (n=3), the only tk condition from which it was possible to induce HLA-A2<sup>HA-1</sup> tetramer-binding CD8<sup>+</sup> T lymphocytes with efficiency comparable to unmodified T cells was B+IL-7/IL-15 (Figure 1, panel a and Table I). After isolation by flow cytometry and polyclonal stimulation with PHA, tk<sup>+</sup> HLA-A2<sup>HA-1</sup> tetramer-binding CD8<sup>+</sup> T cells from the B+IL-7/IL-15 condition expanded and were cytotoxic against targets naturally expressing HA-1 (Figure 1, panel a). To exclude that these findings were restricted to the mH antigen HA-1, similar experiments were carried out with T cells from HLA-A2<sup>pos</sup>/H-Y<sup>neg</sup> (female) donors (n=4), using H-Y as an alternative model alloantigen. Again, the only tk condition in which the efficiency of H-Y-specific CTL induction was comparable to unmodified T cells was B+IL-7/IL-15 (Figure 1, panel b and Table I). In summary, these results show that RV-mediated tk transduction after CD28 costimulation with beads and culturing with IL-7/IL-15 preserves

mH antigen-specific CTL alloreactivity in vitro. Consequently, all subsequent experiments were performed with  $tk^+$  T cells generated with B+IL-7/IL-15.



**Figure 1. Expansion and cytotoxicity of human  $tk^+$  mH antigen-specific CTLs.** (a) The transduction efficiency (left panels) of peripheral blood lymphocytes (PBL) from HLA-A2<sup>pos</sup>/HA-1<sup>neg</sup> donors after either activation with an anti-CD3 mAb (OKT3) and culture with IL-2 or after activation with CD3/CD28-beads and culture with IL-2 (B+IL-2), IL-7 (B+IL-7) or a combination of IL-7 and IL-15 (B+IL-7/IL-15) was evaluated by flow cytometry and is shown as the percentage of T cells positive for the NGFR marker gene (y axis) over the FSC (x axis). Flow cytometry was also used to measure (middle panels) and to isolate (right panels) CD8<sup>+</sup> T cells (x axis) binding the HLA-A2<sup>HA-1</sup> tetramer (y axis). In the case of RV-mediated transduction, HLA-A2<sup>HA-1</sup> tetramer binding CD8<sup>+</sup> T cells were gated for NGFR expression. Insert fold numbers indicates the expansion rate after PHA stimulation. Cytotoxicity is expressed as the percentage of lysis (y axis) at different effector-to-target ratios (x axis) against a HLA-A2<sup>pos</sup>/HA-1<sup>neg</sup> lymphoblastoid cell line (HA1<sup>neg</sup> LCL, white diamonds) or a HLA-A2<sup>pos</sup>/HA1<sup>pos</sup> LCL (HA1<sup>pos</sup> LCL, black diamonds). The cytotoxicity of a HA-1-specific CTL clone (HA1 clone) against the HA1<sup>pos</sup> LCL is shown as reference (white squares). Maximal background cytotoxicity of the HA1 clone against the HA1<sup>neg</sup> LCL was always <5% (not shown). Results from one donor representative of n=3 are shown. (b) The same experimental procedure was applied to HLA-A2<sup>neg</sup>/H-Y<sup>neg</sup> (female) donors using appropriate reagents and control cell lines. Results from one donor representative of n=4 are shown. (c) The expansion of HA-1-specific CTLs (HA1 CTLs, left panel) generated after either two rounds (HA1<sup>x2</sup>, white triangles) or 4 rounds of antigenic stimulation (HA1<sup>x4</sup>, black triangles) is expressed as fold growth. The cytotoxicity of HA1 CTLs (right panel) generated after either HA1<sup>x2</sup> (white diamonds) or HA1<sup>x4</sup> (black diamonds) is expressed as a relative percentage over that of the HA1 clone (see Methods). Each symbol represents the result from a single donor. (d) The expansion (left panel) and cytotoxicity (right panel) of HA-1-specific CTLs generated from B+IL-7/IL-15  $tk^+$  T cells (HA1-tk CTLs) after either HA1<sup>x2</sup> or HA1<sup>x4</sup> are shown. Each symbol represents the result from a single donor. (e and f) Experiments were repeated with H-Y-specific CTLs generated from PBL (HY CTLs) or from B+IL-7/IL-15  $tk^+$  cells (HY-tk CTLs) of HLA-A2<sup>neg</sup>/H-Y<sup>neg</sup> donors after either two (HY<sup>x2</sup>) or four (HY<sup>x4</sup>) rounds of antigenic stimulation, using appropriate reagents and control cell lines. Expansion (left panels) and cytotoxicity (right panels) are shown. Each symbol represents the result from a single donor. Results from a paired t test statistical analysis are shown (\*P<0,05, \*\*P<0,01).

### **Antigen-specific enrichment of human $tk^+$ mH antigen-specific CTLs *in vitro* associates with loss of proliferative potential and acquisition of effector function**

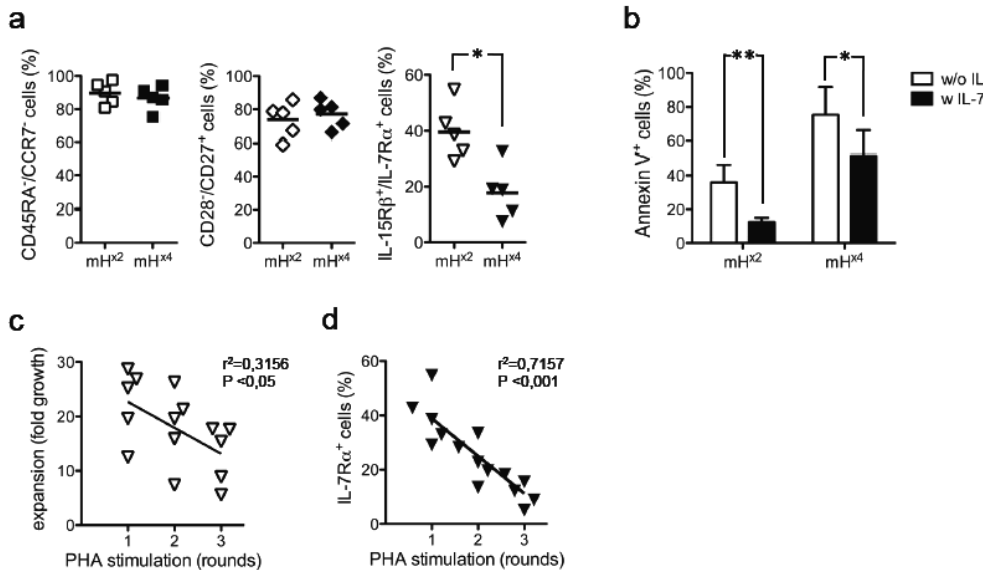
With the aim to generate large numbers of CTLs for further studies, after induction, mH antigen-specific  $tk^+$  CTLs were exposed to additional rounds of antigenic stimulation. Similar to previous studies<sup>27</sup>, this led to a significant enrichment of CD8<sup>+</sup> T cells binding the relevant tetramer. Namely,  $tk^+$  HLA-A2<sup>HA-1</sup> tetramer-binding CD8<sup>+</sup> T cells increased from mean 3,1% (range 2,8 - 4,1) after two rounds of HA-1-specific stimulation to mean 7,2% (range 5.0 – 8.2) after 4 rounds ( $P < 0.05$ ) and  $tk^+$  HLA-A2<sup>H-Y</sup> tetramer-binding CD8<sup>+</sup> T cells increased from mean 2,4% (range 2.1 – 4.7) after two rounds of H-Y-specific stimulation to mean 6.1% (range 4.8 – 7.5) after 4 rounds ( $P < 0.01$ ). Both  $tk^+$  HLA-A2<sup>HA-1</sup> and HLA-A2<sup>H-Y</sup> tetramer-binding CD8<sup>+</sup> T cells isolated by flow cytometry after 4 rounds of stimulation expanded less than cells isolated after two rounds, but displayed a higher degree of cytotoxicity against the relevant mH antigen (Figure 1, panel d and f). The phenomenon was also evident in the case mH antigen-specific tetramer-binding CD8<sup>+</sup> T cells generated from unmodified T cells (Figure 1, panel c and e). Altogether, these results indicate that antigen-specific enrichment of  $tk^+$  mH antigen-specific CTLs *in vitro* associates with loss of proliferative potential and acquisition of effector function.

### **Loss of proliferative potential of human $tk^+$ mH antigen-specific CTLs *in vitro* associates with loss of functional IL-7 receptor expression**

Since the loss of proliferative potential could be related to a progression in T-cell differentiation, before or after antigen-specific enrichment, the phenotype of mH antigen-specific  $tk^+$  CTLs was compared. Before or after the procedure,  $tk^+$  mH antigen-specific CTLs were homogeneously negative for CD45RA and CCR7, i.e. they belonged to the putative T<sub>EM</sub> cell subset (Figure 2, panel a). Moreover, they were indifferently positive for the costimulatory receptor CD27, but negative for CD28 (Figure 2, panel a). However, whilst before enrichment  $tk^+$  mH antigen-specific CTLs co-expressed the  $\alpha$  subunit of the IL-7 receptor (IL-7R $\alpha$ ) and the  $\beta$  subunit of the receptor for IL-2/IL-15 (IL-15R $\beta$ ), after enrichment, there was a selective depletion of cells expressing the  $\alpha$  subunit of the receptor for IL-7 (IL-7R $\alpha$ , Figure 2, panel a).

Elaborating on the assumption that the difference in IL-7R $\alpha$  expression had a functional relevance, the effect of exogenous IL-7 on the ability of  $tk^+$  mH antigen-specific CTLs isolated before or after antigen-specific enrichment to survive upon antigen deprivation was compared *in vitro*. After one week of culture in the absence of antigen, the majority of  $tk^+$  mH antigen-specific CTLs isolated after enrichment was in apoptosis, as demonstrated by annexin V staining, while a considerable fraction of CTLs isolated before enrichment was still viable (Figure 2, panel b). The addition of IL-7 almost completely rescued  $tk^+$  mH antigen-specific CTLs isolated before enrichment, but failed in the case of CTLs isolated after enrichment (Figure 2, panel b).

With the hypothesis that the loss of proliferative potential associated with the loss of IL-7 receptor expression, before antigen-specific enrichment,  $tk^+$  mH antigen-specific CTLs were isolated, exposed to weekly rounds of polyclonal stimulation and analyzed for IL-7R $\alpha$  expression. The expansion rate (Figure 2, panel c) as well as the proportion of  $tk^+$  mH antigen-specific CTLs expressing IL-7R $\alpha$  (Figure 2, panel d) progressively decreased with the rounds of stimulation.

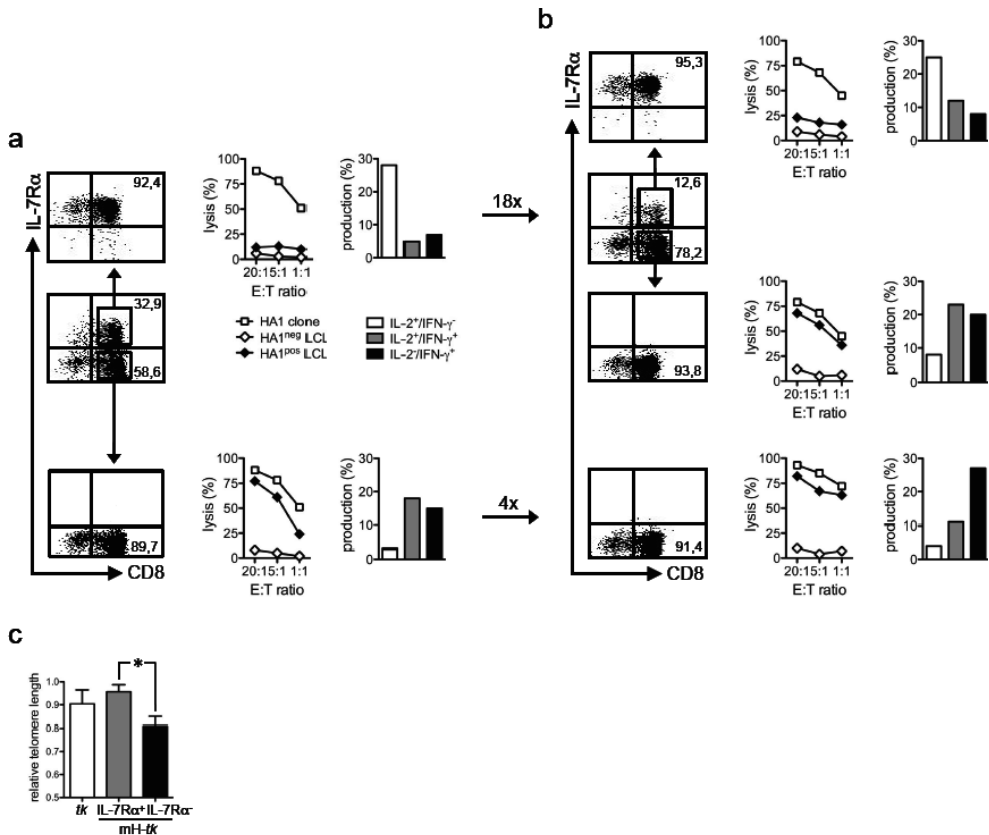


**Figure 2. Differentiation phenotype and IL-7 responsiveness of human  $tk^+$  mH antigen-specific CTLs.** (a) The differentiation phenotype of  $tk^+$  mH antigen-specific CTLs after either two ( $mH^{x2}$ ) or four rounds ( $mH^{x4}$ ) of antigenic stimulation was evaluated by flow cytometry and is expressed as the percentage of CD45RA<sup>+</sup>/CCR7<sup>+</sup> cells (left panel), of CD28<sup>+</sup>/CD27<sup>+</sup> (middle panel) and of IL-15Rβ<sup>+</sup>/IL-7Rα<sup>+</sup> cells (right panel). Each symbol represents the result from a single donor of a group of n=2 HLA-A2<sup>pos</sup>/HA-1<sup>neg</sup> and n=3 HLA-A2<sup>pos</sup>/H-Y<sup>neg</sup> donors. Results from a t test paired statistical analysis are shown (\* $P < 0,05$ ). (b) The percentage of either  $mH^{x2}$  or  $mH^{x4}$   $tk^+$  mH antigen-specific CTLs binding annexin V after culture in the absence of antigen and in the presence (black bars) or in the absence (white bars) of exogenous IL-7 was evaluated by flow cytometry. Means  $\pm$  SD of the data from n=2 HLA-A2<sup>pos</sup>/HA-1<sup>neg</sup> and n=2 HLA-A2<sup>pos</sup>/H-Y<sup>neg</sup> donors and results from a paired t test statistical analysis are shown (\* $P < 0,05$ , \*\*\* $P < 0,005$ ). (c) The expansion of  $mH^{x2}$   $tk^+$  mH antigen-specific CTLs after multiple, weekly rounds of stimulation with PHA (x axis) is expressed as fold growth (y axis). (d) The percentage of IL-7Rα<sup>+</sup> cells (y axis) was evaluated by flow cytometry before each round of PHA stimulation (x axis). Results from linear regression analysis are shown (exact P values).

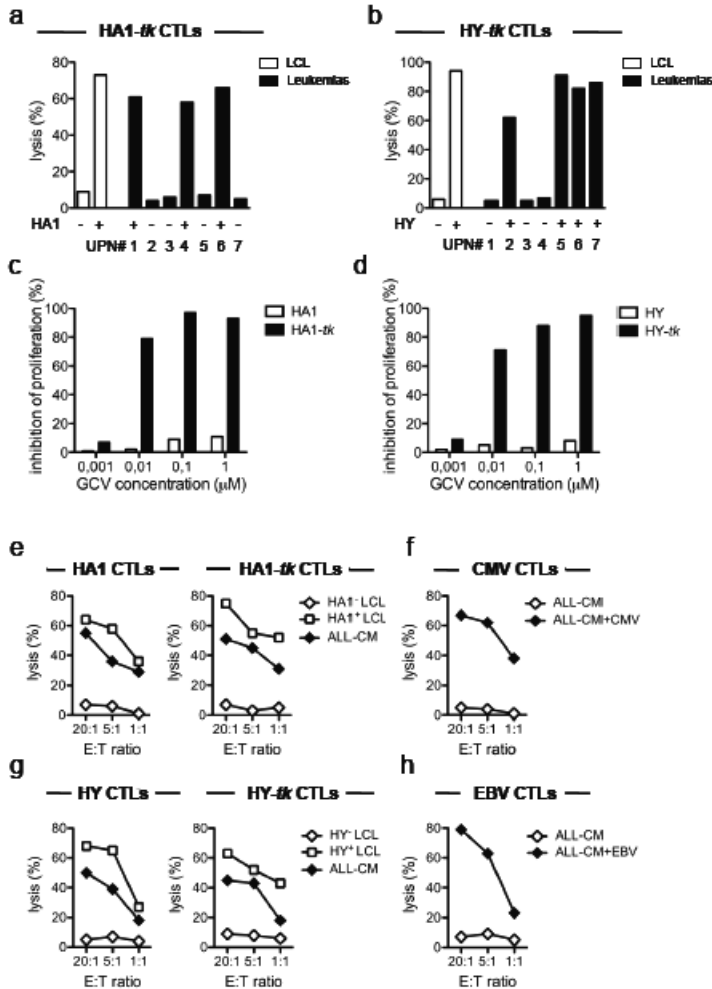
### IL-7 receptor expression identifies human $tk^+$ mH antigen-specific CTLs capable of self-renewal and differentiation into effectors in vitro

To confirm that the loss of IL-7 receptor expression was linked to the acquisition of effector function, experiments based on sequential sorting were performed. Before antigen-specific enrichment,  $tk^+$  mH antigen-specific CTLs were sorted for IL-7Rα expression and analyzed for cytotoxicity and cytokine production. Interestingly, while IL-7Rα<sup>+</sup> cells were poorly cytotoxic and mainly produced IL-2, IL-7Rα<sup>-</sup> cells were highly cytotoxic and essentially produced IFN-γ (Figure 3, panel a and Table II). Upon polyclonal stimulation, IL-7Rα<sup>+</sup> cells expanded and gave rise to a mixed progeny of IL-7Rα<sup>+</sup> and IL-7Rα<sup>-</sup> cells (Figure 3, panel b and Table II). In contrast, IL-7Rα<sup>-</sup> cells did not expand substantially and homogeneously lacked IL-7Rα expression (Figure 3, panel b and Table II). After expansion,  $tk^+$  mH antigen-specific CTLs were re-sorted for IL-7Rα expression and functional testing revealed the same differences in cytotoxicity and cytokine production between IL-7Rα<sup>+</sup> and IL-7Rα<sup>-</sup> cells. Interestingly, flow-cytometry analysis of mean telomere length of  $tk^+$  mH antigen-specific CTLs after the first sorting revealed that IL-7Rα<sup>+</sup> cells had longer telomeres than IL-7Rα<sup>-</sup> cells (Figure 3, panel c), implying that the loss of proliferative potential associated with IL-7 receptor loss coincided with telomere erosion. In

summary, these results suggest that IL-7 receptor expression identifies  $tk^+$  mH antigen-specific CTLs capable of self-renewal and differentiation into effectors in vitro.



**Figure 3. Self-renewal and differentiation of human  $tk^+$  mH antigen-specific CTLs based on IL-7R $\alpha$  expression.** (a) HA1-specific CTLs generated after two rounds of antigenic stimulation were sorted for IL-7R $\alpha$  expression by flow cytometry (left panels). The percentage of CD8<sup>+</sup> T cells (x axis) positive for the IL-7R $\alpha$ (y axis) is shown before and after sorting (arrows). Cytotoxicity (middle panels) is expressed as the percentage of lysis (y axis) at different effector-to-target ratios (x axis) against a HLA-A2<sup>pos</sup>/HA1<sup>neg</sup> lymphoblastoid cell line (HA1<sup>neg</sup> LCL, white diamonds) or a HLA-A2<sup>pos</sup>/HA1<sup>pos</sup> LCL (HA1<sup>pos</sup> LCL, black diamonds). The cytotoxicity of a HA1-specific CTL clone (HA1 clone) against the HA1<sup>pos</sup> LCL is shown as reference (white squares). Maximal background cytotoxicity of the HA1 clone against the HA1<sup>neg</sup> LCL was always <5% (not shown). Cytokine production (right panels) was evaluated by flow cytometry after antigenic stimulation (see Methods) and is expressed as the percentage of cells with the following profile of intracytoplasmic staining: IFN- $\gamma$ /IL-2<sup>-</sup> (white bars), IFN- $\gamma$ /IL-2<sup>+</sup> (grey bars), IFN- $\gamma$ /IL-2<sup>-</sup> (black bars). Insert fold numbers indicate the expansion rate of sorted populations after PHA stimulation. (b) IL-7R $\alpha$  expression (left panels), cytotoxicity (middle panels) and cytokine production (right panels) of the progeny of IL-7R $\alpha^+$  HA-1-specific CTLs cells before and after re-sorting (arrows). After re-sorting, cells were re-stimulated with PHA. Fold growth of IL-7R $\alpha^+$  cells from IL-7R $\alpha^-$  cells was 10.4, fold growth of IL-7R $\alpha^-$  cells from IL-7R $\alpha^+$  cells was 4.7 and fold growth of IL-7R $\alpha^-$  from IL-7R $\alpha^+$  cells was 2.3. Results from the representative donor HA1-tk #1 are shown. (c) Relative telomere length (y axis) of  $tk^+$  T cells generated with CD3/CD28-beads and IL-7/IL-15 (tk, white bar) and of  $tk^+$  mH antigen-specific CTLs (mH-tk) sorted as IL-7R $\alpha^+$  (grey bars) or IL-7R $\alpha^-$  (black bars) was measured by flow FISH (see Methods). Means  $\pm$  SD of relative telomere length from n=2 HLA-A2<sup>pos</sup>/HA-1<sup>neg</sup> and n=1 HLA-A2<sup>pos</sup>/H-Y<sup>neg</sup> donors and results from a paired t test statistical analysis are shown (\*P<0,05).



**Figure 4. Cytotoxicity of tk<sup>+</sup> mH antigen-specific CTLs against leukemia and suicide induction by GCV administration in vitro.** (a) The cytotoxicity of tk<sup>+</sup> HA-1-specific CTLs (HA1-tk CTLs) generated from a HLA-A2<sup>pos</sup>/HA-1<sup>neg</sup> representative donor (HA1-tk #1) was evaluated against different HLA-A2<sup>pos</sup> targets, including lymphoblastoid cell lines (LCL, open bars) and patient-derived leukemic cells typed for HA-1 (negative if R/R, positive if H/R or HH) and is expressed as the percentage of lysis at a 20:1 effector-to-target ratio. (b) The cytotoxicity of tk<sup>+</sup> H-Y-specific CTLs (HY-tk CTLs) generated from a HLA-A2<sup>pos</sup>/H-Y<sup>neg</sup> representative (female) donor (HY-tk #1) was evaluated against different HLA-A2<sup>pos</sup> targets, including lymphoblastoid cell lines (LCL, open bars) and leukemic cells derived from female (HY negative) or male (HY positive) patients and is expressed as the percentage of lysis at a 20:1 effector-to-target ratio. (c) Suicide induction on HA1 and HA1-tk CTLs at increasing concentrations of GCV (x axis) is expressed as GCV sensitivity (y axis, see Methods). Results from the HA1-tk #1 donor are shown. (d) Suicide induction was also assayed on HY and HY-tk CTLs. Results from the donor HY-tk #1 donor are shown. (e) The cytotoxicity of HA1-specific CTLs generated from either PBL (HA1 CTLs) or from tk<sup>+</sup> T cells (HA1-tk CTLs) of the HA1-tk #1 donor is expressed as the percentage of lysis (y axis) at different effector-to-target ratios (x axis) against a HLA-A2<sup>pos</sup>/HA1<sup>neg</sup> lymphoblastoid cell line (HA1<sup>neg</sup> LCL, white diamonds), a HLA-A2<sup>pos</sup>/HA1<sup>pos</sup> LCL (HA1<sup>pos</sup> LCL, white squares) or ALL-CM leukemic cells (black diamonds), which are HLA-A2<sup>pos</sup>/HA-1<sup>pos</sup>/H-Y<sup>pos</sup>. (f) The cytotoxicity of CMV-specific CTLs (CMV CTLs) generated from PBL of the HA1-tk #1 donor is expressed as the percentage of lysis (y axis) at different effector-to-target ratios (x axis) against ALL-CM leukemic cells loaded (black diamonds) or not (white diamonds) with the CMV peptide used for generation. (g) H-Y-specific CTLs were generated from PBL (HY CTLs) and from tk<sup>+</sup> T cells (HY-tk CTLs) of the HY-tk #1 donor and characterized for cytotoxicity against leukemia using appropriate reagents and control cell lines (d) EBV-specific CTLs (EBV CTLs) were generated from PBL of the HY-tk #1 donor and characterized.

**Table II.** Cytotoxicity and expansion of human tk<sup>+</sup> mH antigen-specific CTLs after IL-7R $\alpha$  sequential sorting

<i><sup>a</sup>CTLs</i>	<i><sup>b</sup>IL-7R<math>\alpha</math><sup>+</sup></i> (%)	<i>Sorting</i>			<i><sup>b</sup>IL-7R<math>\alpha</math><sup>+</sup></i> (%)	<i>Re-sorting</i>		
		<i><sup>c</sup>IL-7R<math>\alpha</math></i> (%)	<i><sup>d</sup>Ctx.</i> (%)	<i><sup>e</sup>Exp.</i> (fold)		<i><sup>c</sup>IL-7R<math>\alpha</math></i> (%)	<i><sup>d</sup>Ctx.</i> (%)	<i><sup>e</sup>Exp.</i> (fold)
<i>HA1-tk #1</i>	32,9	92,4 <sup>pos</sup> <1 <sup>neg</sup>	12,7 65,7	17,6 4,1	12,6	95,3 <sup>pos</sup> <1 <sup>neg</sup>	21,2 78,5	10,4 4,7
<i>HA1-tk #2</i>	54,6	86,7 <sup>pos</sup> 2,3 <sup>neg</sup>	16,8 54,2	24,2 4,6	10,9	91,7 <sup>pos</sup> 1,8 <sup>neg</sup>	11,5 84,5	14,5 -
<i>HY-tk #1</i>	21,7	81,8 <sup>pos</sup> 5,6 <sup>neg</sup>	21,2 74,0	35,1 8,7	15,4	89,6 <sup>pos</sup> 1,5 <sup>neg</sup>	22,6 81,2	12,3 3,5

<sup>a</sup>Thymidine kinase-positive CTLs used for sorting: HA1-tk #1, specific for HA-1 and induced from a HLA-A2<sup>pos</sup>/HA-1<sup>neg</sup> donor, HA1-tk #2, specific for HA-1 and induced from a second HLA-A2<sup>pos</sup>/HA-1<sup>neg</sup> donor, HY-tk #1, specific for H-Y and induced from a HLA-A2<sup>pos</sup>/H-Y<sup>neg</sup> (female) donor

<sup>b</sup>Thymidine kinase-positive mH antigen-specific CTLs expressing IL-7R $\alpha$

<sup>c</sup>Thymidine kinase-positive mH antigen-specific CTLs expressing IL-7R $\alpha$  in the positive (pos) and in the negative (neg) fraction after IL-7R $\alpha$  sorting

<sup>d</sup>Cytotoxicity of IL-7R $\alpha$ <sup>+</sup> and IL-7R $\alpha$ <sup>-</sup> tk<sup>+</sup> mH antigen-specific CTLs against a LCL expressing the relevant mH antigen at a 20:1 ratio

<sup>e</sup>Expansion of IL-7R $\alpha$ <sup>+</sup> and IL-7R $\alpha$ <sup>-</sup> tk<sup>+</sup> mH antigen-specific CTLs

### Human tk<sup>+</sup> mH antigen-specific CTLs are cytotoxic against leukemia and sensitive to suicide induction by prodrug administration in vitro

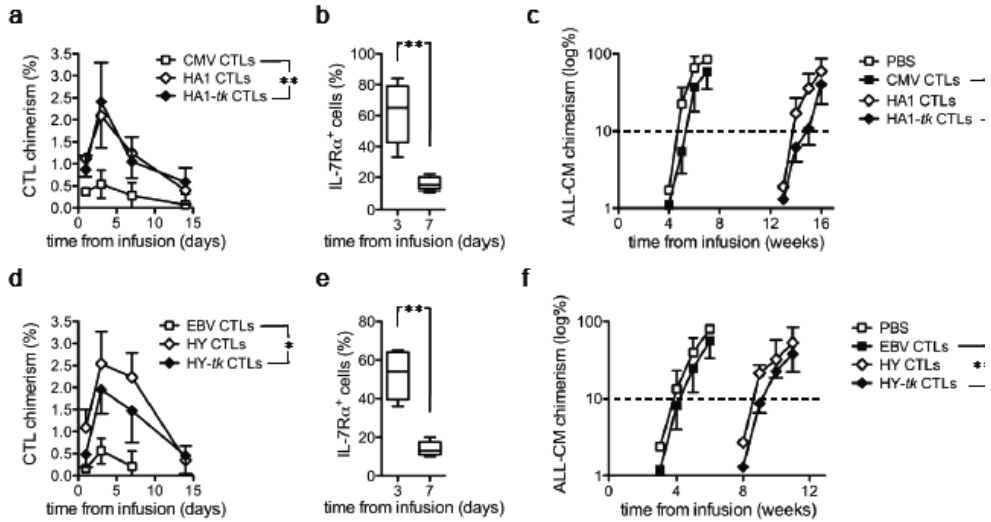
Evidently, demonstrating the antileukemia efficacy of tk<sup>+</sup> mH antigen-specific CTLs would underscore the clinical relevance of our findings. For this purpose, tk<sup>+</sup> mH antigen-specific CTLs were evaluated for cytotoxicity against a panel of HLA-A2<sup>pos</sup> leukemic cells derived from patients (Supplementary Table I). Thymidine kinase-positive HA-1-specific CTLs were cytotoxic against HA-1<sup>pos</sup> leukemic cells derived from patients with different types of leukemia, but not against HA-1<sup>neg</sup> leukemic cells (Figure 4, panel a and Supplementary Figure 1, panel a). By analogy, tk<sup>+</sup> H-Y-specific CTLs were cytotoxic against leukemic cells of male origin (H-Y<sup>pos</sup>) but not against those of female origin (H-Y<sup>neg</sup>, Figure 4, panel b and Supplementary Figure 1, panel b). After demonstration of the antileukemia efficacy of tk<sup>+</sup> mH antigen-specific CTLs in vitro, the functionality of the suicide gene machinery was investigated. As expected, exposing tk<sup>+</sup> but not unmodified mH antigen-specific CTLs to increasing concentrations of the prodrug GCV caused a dose-dependent inhibition of cell proliferation (Figure 4, panel c and d).

### Thymidine kinase-positive mH antigen-specific CTLs display substantial antileukemia efficacy in vivo

In the final part of the study, we aimed at verifying whether suicide-gene modification might decrease the antileukemia efficacy of mHag-specific CTLs in a clinically relevant humanized mouse model<sup>21</sup>. To this aim, different populations of CTLs were generated from two of the HLA<sup>pos</sup> donors previously analyzed. The two donors were chosen as representative for the HA-1 and for the H-Y antigen, respectively. In the case of the HA-1<sup>neg</sup> donor, tk<sup>+</sup> or unmodified HA-1-specific CTLs and control unmodified CMV-specific CTLs were generated after induction with two rounds of antigenic stimulation, isolation and expansion by polyclonal stimulation. Interestingly, tk<sup>+</sup> HA-1-specific CTLs could be generated as efficiently as unmodified HA-1-specific CTLs, confirming that previous RV-mediated tk transduction with B+IL-7/IL-15 was not interfering with the potential for secondary expansion (not shown). Expanded tk<sup>+</sup> and unmodified HA-1-specific CTLs were highly and equally



cytotoxic against ALL-CM leukemic cells (Figure 4, panel e), while CMV-specific CTLs were not, unless leukemic cells were pulsed with the CMV peptide used for generation (Figure 4, panel f). Using the same procedure,  $tk^+$  and unmodified H-Y-specific CTLs were also generated from the H-Y<sup>neg</sup> representative (female) donor and were cytotoxic against ALL-CM leukemic cells, which are of male origin (H-Y<sup>pos</sup>, Figure 4, panel g). As expected, unmodified EBV-specific CTLs generated from the same donor as control, did not recognize ALL-CM leukemic cells, unless these were pulsed with the peptide used for generation (Figure 4, panel h). NOD/scid mice were inoculated with  $10^7$  ALL-CM leukemic cells and, three days later, infused with either  $2 \times 10^7$   $tk^+$  or with unmodified HA-1-specific CTLs. Control mice received saline or  $2 \times 10^7$  CMV-specific human CTLs. HA-1-specific CTLs were detected in the peripheral circulation of all mice (Figure 5, panel a). Circulating  $tk^+$  and unmodified HA-1-specific CTLs peaked at day 3 and persisted up to day 14. CMV-specific CTLs also peaked at day 3, but from day 7 on they were barely detectable (Figure 5, panel a). Interestingly, despite before infusion only 18.3% of  $tk^+$  HA1-specific CTLs expressed IL-7R $\alpha$ , the majority of peaking circulating CTLs were IL-7R $\alpha^+$  (mean 65.1%, range 33 - 84, Figure 5, panel b). This proportion declined over time, possibly indicating ongoing differentiation upon antigen-encounter in vivo. Compared with CMV-specific CTLs,  $tk^+$  and unmodified HA-1-specific CTLs equally and significantly prolonged leukemia-free survival (approximately 9 weeks, Figure 5, panel c,  $P=0.001$ ). In another experiment, after leukemia inoculum, mice were infused with either  $tk^+$  or with unmodified H-Y-specific CTLs. In vivo persistence of  $tk^+$  H-Y-specific CTLs and kinetics of IL-7 receptor expression was similar as in the case of  $tk^+$  HA-1-specific CTLs (Figure 5, panel d and e, IL-7R $\alpha^+$  cells before infusion: 15.5%, on peaking circulating CTLs: mean 52%, range 36 - 65). Compared with the respective control group infused with EBV-specific CTLs,  $tk^+$  H-Y-specific CTLs also significantly prolonged leukemia-free survival, albeit to a slightly lower extent than HA-1-specific CTLs (approximately 5 weeks, Figure 5, panel c,  $P=0.003$ ).



**Figure 5. In vivo persistence and antileukemia efficacy of mH antigen-specific TK<sup>+</sup> CTLs in NOD/scid mice.** (a) At different time points (x axis) after ALL-CM leukemia inoculum and later infusion of unmodified CMV-specific CTLs (CMV CTLs, black squares), unmodified (HA1 CTLs, white diamonds) or tk<sup>+</sup> HA-1-specific CTLs generated from the HA1-tk #1 donor, CTL chimerism (y axis, see Methods) was evaluated in the peripheral blood of individual NOD/scid mice by flow cytometry. Control mice were infused with PBS (white squares). Means  $\pm$  SD of the data from n=5 mice/group and results from an unpaired Mann-Whitney u test statistical analysis are shown (\*\*P<0,01 for CTL chimerism at day 3). (b) At day 3 and 7 after infusion, the percentage of circulating tk<sup>+</sup> HA-1-specific CTLs expressing IL-7R $\alpha$  (y axis) was evaluated by flow cytometry. (c) At different time points (x axis), mice were also individually monitored for ALL-CM chimerism (y axis). Mean log percentages of circulating leukemic cells from n=5 mice/group  $\pm$  SD (y axis) are shown (\*\*\*P<0,005). (d, e and f) CTL chimerism, IL-7R $\alpha$  expression and ALL-CM chimerism was also monitored in mice inoculated with leukemia and later infused with unmodified EBV-specific CTLs (EBV CTLs, black squares), unmodified (HY CTLs, white diamonds) or tk<sup>+</sup> HY-specific CTLs generated from the HY-tk #1 donor (\*P<0,05 for CTL chimerism at day 3, \*\*P<0,01 \*\*\*P<0,005).

## DISCUSSION

In this study, we demonstrate that CD28 costimulation through CD3/CD28-beads and culturing with a combination of IL-7 and IL-15 are necessary and sufficient for the RV-mediated tk transduction of human T lymphocytes retaining mH antigen-specific CTL alloreactivity. Moreover, we provide direct proof-of-concept of the *in vitro* and *in vivo* antileukemia efficacy of mH antigen-specific CTLs derived from tk<sup>+</sup> T cells. Interestingly, during the process of *in vitro* expansion, we observed that tk<sup>+</sup> mH antigen-specific CTLs progressively lost proliferative potential and reciprocally acquired effector function. Within the bulk population, we identified a subset of tk<sup>+</sup> mH antigen-specific CTLs characterized by the highest proliferative potential and by the expression of the IL-7 receptor. This subset could self-renew and give rise to a progeny of effectors, i.e. properties of putative memory stem T cells.

At present, the reason why tk<sup>+</sup> human T lymphocytes generated after CD28 costimulation with beads and culturing with IL-7/IL-15, but not with the other combinations tested, retain mH antigen-specific CTL alloreactivity is unknown. The different cytokines belonging to the  $\gamma$ -chain family apparently have distinct roles in T-cell responses. IL-2 and IL-15, for example, are potent T-cell growth factors that share the IL-2/IL-15 receptor  $\beta$ . As opposed to IL-2, IL-15 does not prime T cells for activation-induced cell death<sup>28</sup> and strongly contrasts regulatory T cells<sup>29</sup>. Importantly, IL-7 is far less potent than IL-2 and IL-15 at inducing T-cell proliferation, but appears to be critically involved in memory T-cell survival<sup>30</sup>. The preservation of mH antigen-reactivity may be explained by a combined action of the two cytokines, which could specifically promote the proliferation and the survival of suicide gene-modified precursors for mH antigen-specific CTLs.

The progressive loss of proliferative potential and the reciprocal acquisition of effector function upon *in vitro* expansion of tumor-reactive CD8<sup>+</sup> T lymphocytes has been already observed in mouse models of adoptive T-therapy of melanoma, and has been shown to associate with the transition from T<sub>CM</sub> to T<sub>EM</sub> cells<sup>31</sup>. We recently described that CD28 costimulation with beads prior to RV-mediated tk transduction enriches for human T<sub>CM</sub> cells, regardless of the cytokine used for culture<sup>18</sup>. Rather unexpectedly, despite their predominant T<sub>EM</sub> phenotype, tk<sup>+</sup> mH antigen-specific CTLs could undergo multiple rounds of expansion *in vitro*, apparently without losing antileukemia efficacy after subsequent transfer *in vivo*. This is in agreement with the observation in a primate model that virus-specific CD8<sup>+</sup> T<sub>EM</sub> clones generated from T<sub>CM</sub>, but not from T<sub>EM</sub> cells, maintain the functional characteristics of the population of origin<sup>32</sup>.

Differently from the linear model of T-cell memory, which assumes a continuous transition between T<sub>NA</sub>, memory and effector T cells, the stem-cell model proposes that antigen encounter determines the early generation of a long-lived putative memory stem T cell characterized by the ability to self-renew and differentiate into memory T cells and effectors<sup>33</sup>. In a mouse model of GvHD, CD8<sup>+</sup> T cells primed *in vitro* for a short period of time with host antigens in the presence of IL-15, were able to persist after adoptive transfer throughout the disease course and to give rise to different T-cell types, including effectors<sup>34</sup>. In analogy, after antigenic stimulation, we found that a subpopulation of tk<sup>+</sup> mH antigen-specific CTLs, identified by the expression of the IL-7 receptor, was characterized by the highest proliferative potential and by the capability to self-renew and to differentiate into potent antileukemia effectors. These characteristics match with those of memory stem T cells. Signaling through Wnt has been recently shown to be involved in the generation of CD8<sup>+</sup> memory stem T cells in mice<sup>35</sup>. It would be interesting in the future to verify whether Wnt signaling also plays a role in the generation of gene-modified memory stem T cells in humans.

Our observation of shorter telomeres in cells losing IL-7 receptor expression suggests that the differentiation of  $tk^+$  mH antigen-specific CTLs into full effectors is unidirectional, as erosion of telomeres in non-malignant cells is thought to be irreversible (discussed in<sup>36</sup>). Conversely, the higher proliferative potential of  $tk^+$  mH antigen-specific CTLs maintaining IL-7 receptor expression could explain their selective enrichment early after infusion into NOD/scid mice. Data in the literature support our conclusions. For example, in vivo persistence and antitumor efficacy of melanoma-reactive human  $CD8^+$  T cells associate with both increased IL-7 receptor expression<sup>37</sup> and longer telomeres<sup>38,39</sup>. Moreover, replicative senescence of EBV-specific human CTLs correlates with irreversible IL-7 receptor deficiency, which can be counterfeited by the genetic insertion of the IL-7 receptor<sup>40</sup>. The demonstration of an association between IL-7 receptor expression and memory stem T-cell properties of  $tk^+$  mH antigen-specific CTLs bears important consequences not only to suicide gene therapy applied to HSCT, but clearly also to the general field of adoptive T-cell therapy. Currently, different improved protocols for adoptive T-cell therapy propose multiparametric sorting before ex vivo manipulation, including sorting for  $T_{NA}$  before genetic redirection<sup>41</sup> or  $T_{CM}$  before antigenic stimulation<sup>42</sup>. In alternative, our results indicate the feasibility and the efficacy of a reverse approach, in which starting from an heterogeneous population gene-modified and/or antigen-specific T cells with high proliferative potential are first generated and then sorted according a single, broadly-applicable surface biomarker, namely IL-7Ra. Moreover, our finding of selective in vivo persistence of IL-7Ra<sup>+</sup> T cells after adoptive transfer of unselected T cell provides evidence for the opportunity of boosting ex vivo generated T cells by in vivo administration of recombinant IL-7<sup>43</sup>.

The basic concept of suicide gene therapy applied to HSCT is that, by infusing polyclonal donor T lymphocytes modified with a suicide gene, it is possible to reconstitute the patient with the whole T-cell repertoire of a healthy donor, including T-cell specificities for viral, tumor and alloantigens, while having possibility to switch-off GvHD. Current clinical protocols for RV-mediated transduction critically reduce alloreactivity and the antileukemia efficacy of suicide gene-modified T cells<sup>9</sup>. The final specific aim of this study was to verify whether suicide-gene modification following CD28 costimulation with beads and culturing with IL-7/IL-15 might decrease the antileukemia efficacy of T cells in vivo. This crucial issue was approached in a clinically relevant humanized mouse model of adoptive T-cell therapy of leukemia, in which targeting a single mH antigen with CTLs significantly prolongs disease-free survival<sup>21,44</sup>. In this setting, we found that single mH antigen-specific CTLs, either HA-1 or H-Y, derived from  $tk^+$  T cells were as effective as those from unmodified T cells, therefore indicating that genetic modification didn't interfere with antileukemia efficacy.

In summary, the results of the present study indicate that a protocol including CD28 costimulation with beads and culturing with a combination of IL-7 and IL-15 preserves the antileukemia efficacy of suicide gene-modified T cells, possibly through the direct targeting of a population of precursors for alloreactive memory stem T cells. A phase I/II clinical trial of suicide gene therapy implementing  $tk^+$  T cells generated with this protocol is currently in preparation for the treatment of leukemia patients in the context of HSCT and will be instrumental for the further investigation of IL-7 receptor expression as a biomarker of sustained, yet controllable, CTL alloreactivity in vivo.

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## **AUTHOR CONTRIBUTION**

A.B. and L.H. equally contributed to this study. A.B. designed and performed research, analyzed and interpreted the data, performed statistical analysis and wrote the manuscript; L.H. designed research, analyzed and interpreted the data, and wrote the manuscript; Z.A. performed research and collected the data; B.N. and M.R. provided vital new reagents; S.K. and S.M. designed research; K.F., F.C. and C. Bordignon analyzed and interpreted the data; C. Bonini and E.G. designed research, analyzed and interpreted the data, and wrote the manuscript.

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SUPPLEMENTARY TABLE AND FIGURES

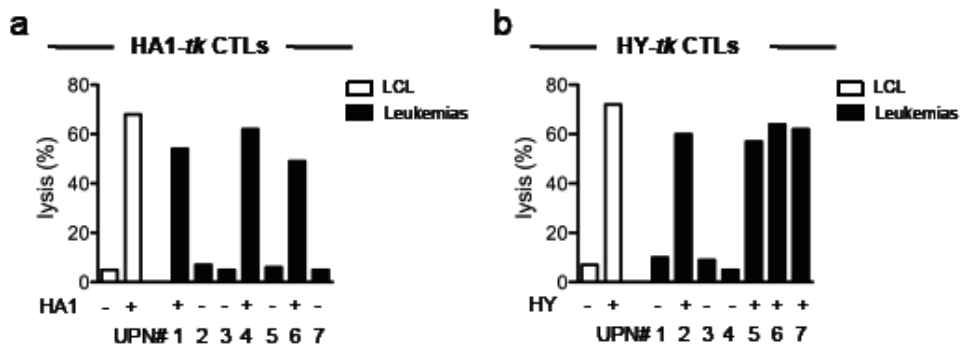
Table S1. Molecular and clinicopathological characteristics of leukemic cells derived from patients

Patient	<sup>a</sup> Diagnosis	<sup>b</sup> FAB subtype	<sup>c</sup> WHO classification	Gender	HLA-A typing	HA-1 typing
UPN #1	AML	M2	AML MD	F	*0201/*2402	H/R
UPN #2	AML	M4	AMML	M	*0201/*1101	R/R
UPN #3	AML	M1	AML without maturation	F	*0201/*0301	R/R
UPN #4	AML	M4	AMML	F	*0201/*2401	H/H
UPN #5	sAML	-	AML MD	M	*0201/*6801	R/R
UPN #6	AML	M2	AML NPM1	M	*0201/*2402	H/H
UPN #7	B-ALL	L1	B-ALL nos	M	*0201/*2402	R/R

a AML, acute myelogenous leukemia, sAML, AML secondary to myelodysplasia, B-ALL, B-cell acute lymphoblastic leukemia

b Subtypes according to the French-American-British (FAB) classification

c Classified according to the World Health Organization (WHO): AML MD, AML with myelodysplasia, AMML, acute myelomonocytic leukemia, AML NPM1, AML with mutated nucleophosmin-1, B-ALL nos, B-ALL non otherwise specified



Supplementary Figure 1. (a) The cytotoxicity of *tk*<sup>+</sup> HA-1-specific CTLs (HA1-*tk* CTLs) generated from another HLA-A2pos/HA-1neg representative donor (HA1-*tk* #2) was evaluated against different HLA-A2pos targets, including lymphoblastoid cell lines (LCL, open bars) and patient-derived leukemic cells typed for HA-1 (negative if R/R, positive if H/R or HH) and is expressed as the percentage of lysis at a 20:1 effector-to-target ratio. (b) The cytotoxicity of *tk*<sup>+</sup> H-Y-specific CTLs (HY-*tk* CTLs) generated from another HLA-A2pos/H-Yneg representative (female) donor (HY-*tk* #2) was evaluated against different HLA-A2pos targets, including lymphoblastoid cell lines (LCL, open bars) and leukemic cells derived from female (HY negative) or male (HY positive) patients and is expressed as the percentage of lysis at a 20:1 effector-to-target ratio.