T Cell Suicide Gene Therapy Prompts Thymic Renewal in Adults after Hematopoietic Stem Cell Transplantation

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ABSTRACT

The genetic modification of T cells with a suicide gene grants a mechanism of control of adverse reactions, allowing safe infusion after partially-incompatible Hematopoietic Stem Cell Transplantation (HSCT). In the TK007 clinical trial 22 adults with hematologic malignancies experienced a rapid and sustained immune recovery after T cell-depleted HSCT and serial infusions of purified donor T cells expressing the Herpes Simplex Virus Thymidine Kinase suicide gene (TK<sup>pos</sup> cells). After a first wave of circulating TK<sup>pos</sup> cells, the majority of T cells supporting long-term immune reconstitution did not carry the suicide gene and displayed high numbers of naïve lymphocytes, suggesting the thymus-dependent development of T cells, occurring only upon TK<sup>pos</sup> cell engraftment. Accordingly, after the infusions we documented an increase in circulating T cell Receptor Excision Circles and CD31+ recent thymic emigrants, and a substantial expansion of the active thymic tissue at chest tomography scans. Interestingly, a peak in the serum level of interleukin-7 was observed after each infusion of TK<sup>pos</sup> cells, anticipating the appearance of newly generated T cells. Taken together, our data show that the infusion of genetically modified donor T cells after transplantation can drive the recovery of thymic activity in adults, leading to immune reconstitution.
INTRODUCTION

Over the last two decades the clinical outcome of Hematopoietic Stem Cell Transplantation (HSCT) registered a constant improvement, availing its role as treatment of choice for high-risk hematological malignancies\(^1\text{-}\text{4}\). Still, even if we overlook the unsolved issue of primary disease recurrence, two intertwined problems keep accounting for most of the treatment-related mortality in transplanted patients: Graft-versus-Host Disease (GvHD) and opportunistic infections. Lethality related to these complications is particularly high in HLA-mismatched transplantation, explaining why alternative HSC sources like Umbilical Cord Blood and haploidentical family donors are only employed in highly specialized centers.

The therapeutic options to treat or prevent GvHD have expanded considerably with the introduction in clinical practice of powerful tools such as infliximab, Anti Thymocyte Globulin (ATG), high-dose cyclophosphamide, and the use of extracorporeal photoapheresis\(^5\text{-}\text{6}\). Still, none of these agents have yet demonstrated a clear-cut clinical benefit in severe steroid-resistant GvHD, and all carry as a drawback the risk of prolonged and profound immune suppression.

Also antimicrobial drugs are still insufficient to treat opportunistic pathogens, and incidence of microbial reactivations and diseases remains high until a functional and complete immune recovery is attained\(^7\). To face this threat, in the last decades, several centers successfully developed approaches of antigen-specific cellular therapies targeting the most lethal pathogens in transplanted patients\(^8\text{-}\text{11}\). However, these targeted adoptive cell therapies require being ready in a short time, are associated with considerable costs, and ultimately grant protection against a limited array of pathogens. Another attractive strategy to improve immune reconstitution after HSCT
is to promote the de novo development of new T cells, often compromised in adult patients by age-related involution of the thymus, which can be aggravated by the concomitant occurrence of GvHD\textsuperscript{12}. The infusion of ex vivo matured T cell precursors and several soluble factors implicated in thymic recovery and T cell maturation (Keratinocyte Growth Factor, Growth Hormone, Interleukin-7 (IL-7)) are currently under evaluation in preclinical and phase I clinical studies\textsuperscript{13-15}.

Given the issues encountered with currently available therapies, several groups have attempted cell processing approaches to manipulate graft features to avoid GvHD occurrence while preserving pathogen-specific T cells. Examples are the photodynamic or pharmacological elimination of allo-specific T cells from the graft\textsuperscript{16-18}, the infusion of products enriched in T regulatory cells\textsuperscript{19} and the ex vivo anergization of T cells, contained in the graft, reactive for patient-specific antigens\textsuperscript{20,21}.

A different strategy have been pursued by our and other groups, with the development of the so called “suicide gene therapy”\textsuperscript{22,23}. In this approach, patients receive purified stem cell grafts followed by the infusions of donor T cells ex vivo manipulated to express an exogenous gene (the “suicide gene”), able to confer sensitivity to specific prodrugs, which activate the gene product and selectively eliminate genetically modified cells. The safety and efficacy of lymphocytes modified by this approach have been tested in several preclinical and clinical studies\textsuperscript{24-26}. In a recent multicentric phase I/II clinical trial (TK007) the infusion of suicide gene-modified T cells after HSCT from HLA-haploidentical family donors provided rapid and effective GvHD control in all patients who required activation of the suicide machinery\textsuperscript{27}. Importantly, most of the patients that received genetically modified T cells achieved rapid T cell immune recovery, with the development of a polyclonal repertoire protective against
pathogens, leading to a significant long-term reduction of infection-related mortality, as compared to what reported after haploidentical HSCT in the absence of T cell addbacks\textsuperscript{28}. We observed that the infusion of the purified gene-modified cells (TK\textsuperscript{pos} cells) was necessary to achieve T cell recovery; however such reconstitution was progressively enriched on T lymphocytes negative for the suicide gene (TK\textsuperscript{neg} cells).

In the present study we grant new insights on the biological events leading to T cell immune reconstitution after TK\textsuperscript{pos} cell infusions, showing an unexpected indirect effect of the gene-modified lymphocytes, which are able to reverse thymic aging and promote the \textit{de novo} generation of T cells from the donor-derived precursors contained in the graft.
MATERIAL AND METHODS

Patients, procedures and biological samples

Fifty-four adult patients with high-risk hematologic malignancies were enrolled to an open, non-randomized, prospective phase I–II clinical trial of haploidentical HSCT and infusions of donor lymphocytes engineered to express the Herpes Simplex Virus-Thymidine Kinase (HSV-TK) suicide gene (TK007 trial). Details on the results of the trial are summarized in a previous report27. Briefly, after a myeloablative conditioning patients received CD34+ peripheral blood HSCs, positively selected to achieve a final median CD34+ count of 11.6×10^6/kg (range 4.6-16.8), with only 1.1×10^4/kg contaminant donor CD3+ cells (range 0.26-10.0). For genetic modification, donor peripheral blood mononuclear cells (PBMCs) were activated with muromonab anti-CD3 in the presence of 600 IU/ml IL-2 (EuroCetus) and transduced with the Replication-Competent Retrovirus (RCR)-free SFCMM-3 retroviral vector to transfer HSV-TK and a truncated form of the Low-affinity Nerve Growth Factor Receptor (ΔLNGFR) marker. After magnetic immune-selection, gene modified T cells were analysed for expression of surface markers, vitality, in vitro sensitivity to activation of suicide gene machinery by ganciclovir (GCV), absence of adventitious agents, replication-competent virus or independent cell growth. Cells with vitality of more than 70%, purity greater than 90%, and which met all the above specifications were released for clinical use. Starting 28 days after HSCT and from the initial dose of 1×10^6 cells/kg (amended during the trial to 1×10^7 cells/kg), infusion of genetically modified T cells were repeated at monthly intervals in the absence of GvHD or immune reconstitution, defined as reaching an absolute CD3 cell count superior to 100/ul in two consecutive samples. None of the patients who achieved immune
reconstitution after TK-cell infusions received systemic administration of IL-2. Peripheral blood samples of patients were collected at serial time-points during treatment follow-up, upon written informed consent approved from the San Raffaele institutional Ethical Committee in accordance with the Declaration of Helsinki.

Flow cytometry analysis
Absolute quantification of circulating T and TK\textsuperscript{pos} cells in TK007 patients was performed according to the ISCT immunological gating protocol\textsuperscript{29}. To assess the phenotype of circulating T cells and the frequency of CD31\textsuperscript{+} Recent Thymic Emigrants (RTEs), patient and healthy donor PBMCs were stained with monoclonal antibodies specific for CD3, CD4, CD8, CD45RA, CD62L, CD31 and LNGFR (all from BD Biosciences). Seven-color immunophenotypic analysis was performed using a Canto II flow cytometer (BD Biosciences) and data were processed using the FCS Express 3.00 software (De Novo Software).

Quantitative PCR for the HSV-TK suicide gene
The presence of the HSV-TK gene sequences was analyzed in genomic DNA extracted from PBMCs by qPCR using the TK-for (GGACACGTTATTTACCCTGTTTCG) and TK-rev (GCCCAGGCAAACACGTTATAC) primers and the FAM-TTGCTGCCCACCAAA-MGB fluorescent probe. Results were normalized upon quantification of the Telomerase reference gene (Forward primer: GGCACACGTTGCTTTTCG; reverse primer: GGTGAACCTCGTAAAGTTTATGCAA; probe: VIC-TCAGGACGTCGAGTGGACACGGTG-TAMRA) and relative expression as
compared to a reference sample (100% of cells positive for HSV-TK) was obtained using the 2-ΔΔCT formula. Sensitivity of the assay allowed detection of up to 1% of HSV-TK positive cells.

Quantification of sjTRECs

Real-time qPCR for single joint T cell Receptor Excision Circles (sjTRECs) was performed as previously described\textsuperscript{30}, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control to standardize DNA content. Briefly, amplification reactions were performed in a final volume of 25 μl containing 50 ng of genomic DNA isolated from PBMCs, TaqMan universal PCR master mix (Perkin Elmer Applied Biosystem), and the appropriate primers and probes. The amount of sjTREC per 100 ng of DNA was determined on the basis of a standard curve developed in-house by cloning the sequence of α1 circles from human cord blood genomic DNA into a plasmid vector, and diluting the plasmid into human DNA from a cell line devoid of TRECs (K562), with a lower limit of detection of 3 copies/100 ng of genomic DNA.

Thymic measurement from chest CT scans

Chest Computed Tomography (CT) scans performed for diagnostic purposes were retrospectively analyzed by two independent experienced radiologists, blinded to the patients’ clinical history, who scored a thymic index based on a semi-quantitative grading scale ranging from 0 (no soft tissue, thymus entirely replaced by fat) to 5 (mass-like appearance, of concern for hyperplasia or thymoma)\textsuperscript{31}.

Quantification of Serum Cytokines
The Bio-Plex Pro Human Cytokine 4-plex array (Bio-Rad) was used to simultaneously analyze the concentrations of IL-2, IL-7, IL-15 and IL-17 in patients’ sera. All samples were analyzed in duplicate. For each studied cytokine a high-sensitivity standard curve was prepared by serial dilutions of recombinant proteins. Data were analyzed using Bio-Plex Manager Version 5.0 software (Bio-Rad).

**Anti-CMV IFN-γ release assay**

T cell specific response against Cytomegalovirus (CMV) of selected patients transplanted from CMV-seropositive donors was assessed by interferon-γ (IFN-γ) Enzyme-Linked Immunosorbent Spot (ELISpot) assay, as described previously. Briefly, IFN-γ release from $10^5$ PBMCs was assessed upon 24-hour incubation with $10^4$ fibroblasts obtained from patient punch-skin biopsies infected with CMV strain AD169. IFN-γ spots were counted using a KS-ELISpot Reader (Zeiss), and specific release was calculated as the number of spots observed in the presence of CMV-infected fibroblasts minus those observed in the presence of uninfected fibroblasts.
RESULTS

Infusion of gene-manipulated donor T cells after transplantation prompts the recovery of a transgene-negative T cell repertoire

Between 2002 and 2008, 54 adult patients underwent HSCT from HLA-haploidentical family donors for high-risk hematologic malignancies in a multicentre, open, non-randomised, prospective phase I–II clinical trial (TK007). The infused graft was extensively selected for CD34+ cells, and contaminating T cells were $1.1 \times 10^4$/kg. To improve immune reconstitution while controlling GvHD patients with documented engraftment of donor HSCs were enrolled to receive TK cells. Excluding criteria for infusion included the presence of CMV reactivations requiring ganciclovir treatment, GvHD or spontaneous T cell reconstitution, which was never observed at the time of the scheduled TK cell infusions. Twenty-eight patients received serial monthly infusions of donor lymphocytes genetically modified to express HSV-TK and ΔLNGFR, starting from day 28 after transplantation. All infused cell products displayed a high purity of TK\textsuperscript{pos} cells (average $94.69 \pm 1.92\%$, n=43; Figure 1A).

Patients who did not receive TK\textsuperscript{pos} cell add-backs failed to attain T cell immune reconstitution (defined as an absolute count of circulating T cells superior to 100/μl), and experienced a dismal clinical outcome, mostly due to infectious complications. Conversely 22/28 patients who received the purified TK\textsuperscript{pos} cells recovered protective T cell counts at a median time of 74 days after transplantation, which were stably maintained over time, with a concomitant improvement in clinical outcome mostly due to reduction of late transplant-related mortality\textsuperscript{27}.

These results demonstrated that the infused TK\textsuperscript{pos} cells were in most treated patients necessary and sufficient to achieve a robust T cell reconstitution. Moreover, post-
transplantation immune recovery of TK007 patients demonstrated persistent high counts of circulating Natural Killer cells and, mostly due to the use of Rituximab as part of the conditioning regimen, low B cell counts, which were eventually recovered at one year after transplantation (Figure 1B). Unexpectedly, during the immunological follow-up of treated patients we detected in the peripheral blood also consistent numbers of CD3+ cells which were negative for the surface marker ΔLNGFR (TK<sup>neg</sup> cells), starting from the time of immune recovery and progressively growing in frequency and absolute counts to dominate the newly reconstituting T cell repertoire (Figure 1C). Hematopoietic chimerism in bone marrow and peripheral blood was full donor for all these patients. Importantly, donor-derived TK<sup>neg</sup> cells could not be detected in patients who did not receive genetically modified T cells, nor in those who did not experience TK<sup>pos</sup> cell engraftment, thus precluding any further analysis on the features of their T cell repertoire.

Quantitative PCR for the HSV-TK gene performed on peripheral blood of treated patients confirmed the results obtained from the immunophenotype, demonstrating a tight correlation with the expression of the surface marker ($r^2=0.597$, $p<0.0001$), excluding the loss, or downregulation, of ΔLNGFR expression on TK<sup>pos</sup> cells (Figure 1D).

TK<sup>neg</sup> cells were enriched in CD4 T cells as compared to their TK<sup>pos</sup> counterparts or their respective infused cell products. Moreover, only in the TK<sup>neg</sup> subset we could detect a recovery of T lymphocytes with a naïve phenotype (CD62L+CD45RA+), which were barely detectable at the time of immune reconstitution (4.25 cells/μl, 3.22% of CD3+ cells), and increased over time to reach physiological cell counts at 1 year after HSCT (250.70 cells/μl, 24.20% of CD3+ cells), of particular relevance in a patient population with the median age of 55 years (range 17-64; Figure 1E).
significant enrichment in γδ T cells nor CD4⁺CD25⁺CD127dimFoxP3⁺ T regulatory cells could be evidenced at any timepoint in TK007 patients as compared to healthy controls (Supplementary Figure 1).

This intriguing observation led us to further investigate the biological origin of TKneg cells and to hypothesize that they might have been originated in the host from the donor progenitors, through a thymic-dependent pathway.

**Appearance of sjTRECs occurs even in elderly patients after TKpos cell add-backs**

SjTRECs are byproducts of the physiological rearrangement of the α chain of the T cell receptor, which are maintained as episomal DNA in newly generated T lymphocytes when they egress from the thymus. Therefore their quantification represents a reliable assessment of thymic output12.

We longitudinally measured by qPCR sjTREC counts in peripheral blood of 13 patients treated with TK cells (Figure 2). As expected from the adult age of the patient cohort, sjTRECs at the time of transplantation were below the detection limit of the method in six out of eight patients (75%). At immune reconstitution sjTRECs were detectable in one single patient (7.7%). This was in line with the low numbers of circulating T cells at this early time-point (14.67% of PBMCs), with a further dilution of the sjTREC-rich naïve subset, a minor fraction of CD3+ cells (Figure 1D). Parallel to the increase in the numbers of naïve T cells also sjTREC counts rose over time, and at one year after HSCT 6/9 patients (66.67%) had detectable circulating sjTRECs, evidencing an important increase in thymic output as compared to their pre-transplantation determination and to an age-matched cohort of patients at one year
after non T cell-depleted haploidentical HSCT. Interestingly 3 out of 6 patients with detectable circulating sjTRECs were older than 50 years of age at time of HSCT.

**Naïve TK<sup>neg</sup> cells reconstituting after suicide gene therapy are Recent Thymic Emigrants**

The surface immunoglobulin-like receptor CD31 (PECAM-1) is a cell adhesion and signaling receptor expressed on hematopoietic and endothelial cells. Amongst CD4+ naïve T cells, positivity for CD31 can distinguish the sjTREC-rich RTEs from aged naïve cells that did not encounter their cognate antigen and underwent homeostatic proliferation<sup>32,33</sup>. The relative size of the CD31+ compartment amongst naïve CD4 cells is an index of the contribution of thymic-dependent lymphopoiesis to the immune repertoire, and decreases with age as thymic involution takes place<sup>34,35</sup>. Since in TK007 treated patients naïve CD4+ cells were detectable at all time-points following TK<sup>pos</sup> cell add-backs (Figure 3A), we analysed CD31 expression to quantify RTEs.

In a control cohort of 26 healthy individuals, CD31 expression in CD4 naïve lymphocytes displayed a stringent correlation with age of the subject ($r^2=0.728$, $p<0.0001$; Figure 3B), with an average frequency of CD31+ RTEs of 58.28±13.48% for adults.

The frequency of CD31+ RTEs measured in 18 adult patients before haploidentical HSCT for hematological malignancies was comparable to that determined in age-matched healthy controls, demonstrating that nor underlying disease nor previous treatment deregulate CD31 expression. Of these 18 patients, 8 were enrolled to the TK007 protocol and received a CD34-selected graft and gene-modified lymphocytes, whereas 10 underwent an unselected, non-T cell-depleted procedure followed by
pharmacological GvHD prophylaxis. As summarized in Figure 3D and exemplified for a representative patient in Figure 3C (UPN#4, age 57), we observed that in TK cell-treated patients almost the totality of TK$^{\text{neg}}$ naïve CD4 cells were positive for CD31, thus *bona fide* RTEs (89.54$\pm$9.55% at immune reconstitution, 81.84$\pm$15.9% at 6 months after HSCT, and 79.55$\pm$16.66% at 12 months after HSCT). Even at 1 year after transplantation, when naïve T cells were fully recovered, more than 80% of these cells were thymic emigrants, indicating the continuous output of cellular elements with novel specificities. Importantly, the control group of patients who underwent unselected HSCT, despite achieving comparable quantitative T cell recovery, with median CD3+ cell counts of 734$\pm$474/µl at 90 days after HSCT, had very low frequencies of RTEs, suggesting that in this group of patients peripheral expansion of donor T cells contained in the graft accounted for immune recovery.

**Chest CT scans of patients treated with TK$^{\text{pos}}$ cells demonstrate regeneration of bioactive thymus**

Irreversible age-dependent fatty involution of thymic epithelia is the main cause of the low output of new T cells in adults. Chest CT scans can distinguish epithelial from adipose tissue, granting a non-invasive and reproducible way to measure the volume of the biologically active thymus$^{31,36}$. The volume of the CT-dense active thymus, expressed semi-quantitatively as Thymic Index (TI), has been shown to significantly decrease over time in healthy subjects, reaching values beneath 1 (minimal soft tissue, barely recognizable) for individuals over 50 years of age.

We retrospectively evaluated chest CT scans performed during the follow-up of 28 patients enrolled to the TK007 study, 21 of whom received one or more infusions of purified TK$^{\text{pos}}$ cells.
Consistent with their age, in most of our patients no thymic soft tissue could be detected before HSCT (average TI 0.48±0.63). The infusion of the stem cell graft had a minimal effect on promoting thymic recovery, since in patients studied before TK⁺ cell infusions no change in thymic index could be evidenced (mean TI 0.75±0.93, p = n.s.). On the contrary, even elderly patients treated with TK⁺ cells showed a consistent increase in the amount of soft tissue detectable by chest CT scan, with de novo appearance of well-defined tissue areas suggestive for bioactive epithelium (mean TI 1.33±1.01, p<0.0001 vs before HSCT, p=0.0039 vs before TK⁺ cell infusion; Figure 4A-E).

These results suggest the regeneration of the physiological thymic tissue following the infusion of donor gene-modified T cells, which is paralleled by the emergence of RTEs and sjTRECs in the peripheral blood.

**Interleukin-7 serum levels peak after TK⁺ cell infusions, preceding T cell reconstitution**

Thymic activity and T cell differentiation are tightly regulated by an asset of concerted signals, including cell-to-cell interactions and soluble factors, amongst which IL-7 has been reported to play a major role. IL-7 is produced by stromal cells in the bone marrow and thymus, where it is required for the development of mature T cells, and has a prominent role in the peripheral compartment, where it promotes the survival and proliferation of naïve and memory T lymphocytes.

We longitudinally measured the serum concentrations of IL-2, IL-7, IL-15, and IL-17 in selected TK007 patients who received TK⁺ cell add-backs.
Neither IL-2 nor IL-17 could be detected in the peripheral blood of patients studied, whereas IL-15 was detectable only for the days immediately following HSCT conditioning, returning to basal levels by day 19 (data not shown).

A similar peak in serum concentration in the early post-transplantation days could be detected for IL-7, possibly induced by the lymphodepletion caused by the conditioning regimen (Figure 5). Strikingly, in most patients who experienced engraftment of the infused TK^pos cells and T cell recovery (7 out of 8 patients studied), an additional sharp increase in the serum levels of IL-7 occurred early after the TK^pos cell add-backs, reaching values not commonly observed in physiological conditions (Figure 5B). Importantly, this second peak in IL-7 serum concentration was often followed by a concomitant rise in peripheral T cell counts, leading to immune reconstitution (dashed line in Figure 5). Interestingly, none of the two patients who failed T cell reconstitution following TK^pos cell infusions showed a rise in IL-7 concentration (Figure 5 D and E), nor did the only patient studied who received an unmanipulated T cell add-back due to impending disease relapse (UPN#9, day 309, Figure 5A).

Newly generated TK^neg cells ensure protective immunity after elimination of TK^pos cells and control of GvHD

One of the major hurdles to the infusion of donor T cells in the context of HSCT from haploidentical donors is the extremely high risk of severe GvHD mediated by alloreactive T lymphocytes\(^40\). This potentially lethal complication often requires prolonged immunosuppressive therapy, in turn leading to loss of immune protection against pathogens and, ultimately, to an increase in infectious morbidity and mortality. In the TK007 clinical trial 11 patients developed GvHD, and all of them
achieved complete resolution of all sign and symptoms by the activation of the suicide gene in TK\textsuperscript{pos} cells through intravenous administration of GCV. As expected, this resulted in a consistent drop in the counts of peripheral blood circulating TK\textsuperscript{pos} cells, with only a minor effect on the numbers of TK\textsuperscript{neg} cells\textsuperscript{27}.

We tested the functional activity of T cells harvested from five TK007 patients who suffered GvHD against CMV, a life-threatening post-transplantation pathogen: in all of them the frequency of T cell capable of releasing IFN-\(\gamma\) in response to CMV was not decreased after the treatment with GCV and elimination of TK\textsuperscript{pos} cells, and was similar to that observed in patients who did not suffer GvHD (Figure 6A).

Importantly, these \textit{ex vivo} observations had a direct clinical counterpart: the frequency of viral reactivations did not rise in TK007 patients who experienced GvHD, remaining as low as in patients who achieved T cell immune reconstitution in the absence of GvHD, whereas patients with no TK\textsuperscript{pos} cell engraftment did not recover their immune repertoire against viruses, and consequently experienced a much higher incidence of viral reactivations (Figure 6B).

Taken together, these data demonstrate that activation of the suicide gene machinery can abrogate GvHD without impairing the physiological immunity against pathogens, ultimately suggesting that, once immune recovery is obtained following TK\textsuperscript{pos} cell infusions, patients may significantly rely on newly generated TK\textsuperscript{neg} cells for long term protection against infections.
DISCUSSION

Early recovery of a competent immune system to protect patients against opportunistic infections is a major open issue in the field of allogeneic HSCT. This is especially true in HLA mismatched HSCT, a context in which donor T cells display an unfavorable balance between their anti-host alloreactivity, main determinant of GvHD, and their physiological activity against pathogens. In the TK007 clinical trial, the infusion of donor T cells expressing the HSV-TK suicide gene after T cell-depleted HSCT promoted rapid immune reconstitution while allowing a safe mechanism of GvHD control in all patients facing this potentially lethal complication (11/11, 100%). In the present study we grant new insights into the immune recovery of TK007 patients, demonstrating that those genetically modified T cells promoted thymic renewal and the de novo development of T cells in adults. Other investigators have evidenced that T cell infusions after HSCT can quicken immune reconstitution by accelerating the recovery of a polyclonal repertoire. The study context offered by TK007 is however quite unique, allowing the in vivo tracking of infused donor T lymphocytes, genetically marked, and their discrimination from other cells of donor origin infused with the graft, negative for the transgene. This peculiar setting allowed us to recognize that the majority of T cells reconstituting after HSCT were not directly derived from peripheral expansion of the infused add-backs, but rather from the graft. Most naïve lymphocytes expressed CD31, a hallmark of RTEs, confirmed by the re-appearance of detectable sjTREC counts and bioactive thymic tissue in adult patients, often older than 50 years. All these evidences point to a thymic origin of the newly reconstituting T cells and help explain the clinical outcome of TK007 patients. Besides an efficient protection against infectious events, an unexpectedly low
incidence of GvHD was recorded in these patients despite the absence of any pharmacological immunosuppressive treatment: we can now speculate that this was due to the fact that the high numbers of T cells circulating in these patients had achieved central tolerance in the patient thymus, with negative selection of host-reactive cells.

Notably, the renewal of thymic activity was a direct consequence of the infusion of TK<sup>pos</sup> cells, as supported by several experimental evidences. First, in the few patients in whom TK<sup>pos</sup> cells were not infused for concomitant clinical reasons, we did not observe T cell recovery, in line with the historical literature data of haploidentical T cell-depleted HSCT, describing a time to T cell reconstitution of up to one year<sup>19,42</sup>. For this reason we could not compare the quality of immune reconstitution obtained upon TK-cell infusions with that observed after T cell depleted haploidentical HSCT at similar time-points. However, comparison with patients undergoing non T-cell depleted HSCT showed that the infusion of mature donor T is not able by itself to enhance RTE generation in the thymus, as demonstrated by the low percentages of CD31<sup>+</sup> CD4 naïve T cells observed in this cohort of patients.

Another evidence supporting the direct role of TK<sup>pos</sup> cell add-backs in prompting thymic activity comes from the novel finding that these infusions are accompanied by a systemic release of IL-7, a relevant player in the early stages of T cell maturation<sup>37</sup>. The observation of the peak in the serum concentration of IL-7 elicited by TK<sup>pos</sup> cells is furthermore intriguing, since it apparently occurred exclusively in those patients in whom the add-backs led to immune reconstitution. Gene expression profiling studies did not evidence IL-7 production nor from retrovirus-transduced T cells nor from TK<sup>pos</sup> cells circulating in treated patients<sup>43,44</sup>, so it should be hypothesized that this effect was achieved through the triggering of additional cellular mediators such as
thymic epithelial cells, able to release IL-7 and propagate the process\textsuperscript{38}. Of notice, IL-7 is not able to induce per se thymic renewal nor thymopoiesis\textsuperscript{15}, so it should not be considered sufficient to attain the final effect of post-transplantation T cell recovery.

Our finding that allogeneic T cell infusions can prompt the renewal of the adult thymus are in apparent contradiction with historical data from animal models, traditionally associating alloresponses with a detrimental effect on thymic output, mainly due to the occurrence of thymic GvHD\textsuperscript{45}. Accordingly, GvHD is usually associated with low sjTREC counts and with a more profound post-transplantation immune deficiency\textsuperscript{6,12}. A possible explanation for the opposite effect mediated by the infusion of TK\textsuperscript{pos} cells comes from the phenotype of the infused lymphocytes: to achieve efficient transduction by the retroviral vector, donor T cells underwent polyclonal stimulation with anti-CD3 antibody and IL-2, shifting their phenotype toward that of effector cells. Experimental transplantation studies demonstrated that the risk of GvHD is mainly correlated to the presence of naïve and central memory cells in the graft, whereas effectors pose a minor threat\textsuperscript{46}. This observation is also in line with the overall low incidence of severe GvHD observed upon the infusions in TK007 patients, despite the high numbers of haploidentical T cells they received\textsuperscript{27}.

Several studies on immune reconstitution after HSCT have consolidated the relevance of recovery of naïve T lymphocytes, polyclonal Vβ repertoire and sjTREC counts as determinants of long-term clinical outcome\textsuperscript{47,48}. It has in fact to be taken into account that transplanted patients face a 20-fold higher risk of infections during their life after HSCT, which is a consistent cause of morbidity and mortality\textsuperscript{49}. TK007 patients display all the features of a robust immune recovery, possibly explained by the continuous output of new T cell specificities, and accordingly no serious infectious
event has been registered in any of the treated patients after day 166 (last death) from HSCT.

Moreover, the process we described is long-lasting and self-maintaining: the frequency and absolute counts of naïve T cells and CD31+ RTEs kept increasing in time. In accordance, serial CT scans obtained from patients after the add-backs showed stability of the active thymic tissue appeared after the infusions. The elucidation of the fine mechanisms underlying the long-term reversal of thymic aging would be a relevant step forward in the field, since most of the molecules currently in clinical use or under study to boost thymopoiesis and ameliorate post-transplantation immunity cease their stimulatory effect once discontinued14.

Finally, the activation of the suicide gene and elimination of TKpos cells in case of GvHD did not come at the price of losing immune competence against pathogens, thus allowing to separate the detrimental GvHD reaction from the desired graft-versus-infection effect. Still, in our view, also the direct contribution of TKpos cells to the outcome of HSCT should be emphasized. In fact, the process of thymic renewal they promote is lengthy and unlikely to protect against pathogens in the early months after transplantation: the infusion of the polyclonal effector TKpos cells may have a major role as first line of defense, bridging to the recovery of the new T cell repertoire matured in the thymus. Moreover, it is unlikely that the newly reconstituted T lymphocytes, possibly tolerized to host antigens during their development, may display antitumor activity: conversely, the emergence of mutant variants of the original leukemia with de novo loss of the patient-specific HLA after serial TKpos cell infusions in two TK007 patients is a strong indirect evidence of the robust antileukemic potential of TKpos cells27,50.
Taken together, our data suggest that thymopoiesis is reactivated upon the infusion of the gene-modified lymphocytes, prompting the generation of a protective T cell compartment, able to reduce the incidence of infectious events following HSCT. Not only a fully competent immune system is re-established, but also the morbidity and mortality related to GvHD is dramatically reduced in patients treated with TKpos cells. The combined effects of reduction of infections and control of GvHD, to which our novel findings grant a biological explanation, ultimately result in a considerable gain in terms of clinical outcome. A multicenter, randomized phase III clinical trial (TK008 study) to assess the efficacy of TKpos cells in the context of haploidentical HSCT for leukemia started in 2010 in Italy and is currently expanding to multiple centers throughout Europe and USA.
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AUTHORSHIP

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FIGURE LEGENDS

Figure 1. Infusion of purified TK<sup>pos</sup> cells after T cell-depleted HSCT prompts the immune recovery of TK<sup>neg</sup> naïve T cells. (A) Composition of 43 genetically modified T cell products infused to 25 patients enrolled to the TK007 clinical trial: upon magnetic selection the large majority of infused cells express the surface marker ΔLNGFR (TK<sup>pos</sup>, in black), whereas only a minor fraction is negative for the transgene (TK<sup>neg</sup>, in white). (B) Absolute counts of circulating T (circles), B (squares), and NK (triangles) lymphocytes in TK007 patients. Shown is average with Standard Deviation (SD). (C) Absolute counts of circulating T cells in TK007 patients before (Baseline) and at different time-points after the infusion of TK<sup>pos</sup> cell add-backs (at immune reconstitution, defined as the attainment of a CD3 cell count above 100 cells/μl; at 6 months after HSCT; and at 12 months after HSCT). Histogram bars are subdivided to represent the relative frequency of circulating TK<sup>pos</sup> (in black) and TK<sup>neg</sup> (in white) T cells at each time-point. Shown is average with SD. (D) Correlation between the frequency of PBMCs expressing on their surface the ΔLNGFR marker (on the x axis) and that of PBMCs having integrated in their genome the HSV-TK transgene, as assessed by specific qPCR (on the y axis), in 25 patients enrolled to the TK007 trial. (E) Absolute counts of circulating TK<sup>pos</sup> (left panels) and TK<sup>neg</sup> (right panels) T cells at different time-points after HSCT and TK<sup>pos</sup> cell add-backs, subdivided according to the CD4 or CD8 subtype (in black and white, respectively; upper panels) and to their naïve, central memory or effector phenotype (in black, grey, and white, respectively; lower panels). Shown are the averages, with SDs, of the results obtained from 10 TK007 patients.
Figure 2. sj TREC\text{s} rise in the peripheral blood of TK007 patients after HSCT and suicide gene therapy. Absolute counts of sjTRECS per 100 ng of genomic DNA extracted from PBMCs of TK007 patients at different time-points during their follow-up and, as control group, from patients who underwent non T cell-depleted haploidentical HSCT. Pie charts below the graph represent the percentage of patients in whom circulating sjTREC counts were above (in black) or below (in white) the sensitivity of the detection method (3 TREC copies/100 ng of DNA).

Figure 3. Naïve T cells circulating after TK\textsuperscript{pos} cell add-backs are CD31\textsuperscript{+} Recent Thymic Emigrants. (A) Frequency of CD4 naïve T cells amongst total CD3\textsuperscript{+} lymphocytes in healthy individuals (white bar) and in TK007 patients before treatment and at different time-points during their follow-up (black bars). Shown is average with SD. (B) Correlation between age of the subject (x axis) and the frequency of CD31\textsuperscript{+} cells amongst the CD4 naïve subset (y axis) in 26 healthy individuals. (C) CD31 expression analysis in a representative patient from the TK007 clinical trial (UPN\#4, age 57). In the upper row are shown the dot plots of CD3\textsuperscript{+}CD4\textsuperscript{+}ΔLNGFR\textsuperscript{-} T cells, according to their expression of CD45RA (horizontal axis) and CD62L (vertical axis) at different time-points during the treatment: gated in black are naïve cells, identified as CD45RA\textsuperscript{+} and CD62L\textsuperscript{+}. Histograms below the dot plots represent CD31 positivity amongst naïve CD4 cells (in black) and, as a negative control, amongst effectors (in white). (D) Frequency of CD31\textsuperscript{+} cells amongst naïve CD4\textsuperscript{+} T lymphocytes in healthy individuals (white diamonds), in TK007 patients at different time-points during their follow-up (black diamonds), or in a control group of patients receiving haploidentical HSCT without T cell depletion of the graft, followed
by pharmacological GvHD prophylaxis (grey diamonds). Shown is average with SD. One asterisk indicates p<0.05, two asterisks p<0.001 in a paired sample Student t test.

**Figure 4. Chest CT scans document an increase in the volume of active thymic tissue after TK\textsuperscript{pos} cell add-backs.** Panels A-D show a detail on the thymic region (indicated by white arrows) from chest CT scans performed for diagnostic purposes in two representative TK007 patients (panel A-B: UPN# 25, age 64; panel C-D: UPN#15, age 17). Note the increase in density in the thymic region in the scans performed after TK\textsuperscript{pos} cell add-backs (B, TI=2, and D, TI=3, performed at 50 and 135 days from last TK\textsuperscript{pos} cell add-back, respectively) as compared to their counterparts before treatment (A and C, both TI=1). Panel E summarizes results obtained from the retrospective analysis of 61 chest CT scans from TK007 patients, each scored a TI value by two independent expert radiologists (y axis). Two asterisks indicate p<0.005, three asterisks p<0.0005 in a paired sample Student t test.

**Figure 5. TK\textsuperscript{pos} cell add-backs prompt the systemic release of IL-7, leading to T cell immune recovery.** Line graphs indicate the serum concentration of IL-7 (solid lines) and the absolute T cell counts (dashed lines) in three representative TK007 patients who experienced systemic engraftment of TK\textsuperscript{pos} cells and subsequent immune reconstitution (three out of twelve studied patients are shown, panels on the left) and in two of the three studied patients that received the suicide gene-modified cells but failed to attain T cell reconstitution (panels on the right). Arrows indicate time of HSCT (white), infusions of TK\textsuperscript{pos} cells (black) or infusions of unmanipulated donor lymphocytes (grey).
Figure 6. Once immune reconstitution is achieved, selective elimination of TK\textsuperscript{pos} cells by activation of the suicide gene does not impair immunity against pathogens. (A) Histogram bars represent IFN-\(\gamma\) release in response to CMV-infected (top section of each bar) or uninfected (bottom section of each bar) fibroblasts by T cells harvested from hematopietic stem cell donors (in black), from TK007 patients that achieved T cell immune reconstitution and did not experience GvHD (in white), and from TK007 patients who suffered GvHD, assessed \textit{ex vivo} before and after the administration of GCV and the resolution of GvHD (in grey). All studied patients were transplanted from CMV-seropositive donors. Shown is average with Standard Error Mean. (B) Frequency of viral reactivations over time after HSCT in patients who received TK\textsuperscript{pos} cells and achieved (solid line, black diamonds), or did not achieve (dashed line, white diamonds) T cell recovery. Grey bars represent the frequency of viral reactivations in the two months preceeding and following GvHD treatment in those TK007 patients in whom this complication occurred. Frequencies are expressed as number of viral reactivations per patient per month.
Figure 2

TREC copies / 100 ng DNA

Pre-HSCT (n=8)
At immune reconstitution (n=13)
At 6 months (n=7)
At 12 months (n=9)
Non T cell-depleted at 12 months (n=5)

sjTREC positivity (%)
T cell suicide gene therapy prompts thymic renewal in adults after hematopoietic stem cell transplantation

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