OncoImmunology

ISSN: (Print) 2162-402X (Online) Journal homepage: https://www.tandfonline.com/loi/koni20

Zoledronic acid boosts γδ T-cell activity in children receiving $\alpha\beta^+$ T and $CD19^+$ cell-depleted grafts from an HLA-haplo-identical donor

A. Bertaina, A. Zorzoli, A. Petretto, G. Barbarito, E. Inglese, P. Merli, C. Lavarello, L. P. Brescia, B. De Angelis, G. Tripodi, L. Moretta, F. Locatelli & I. Airoldi

To cite this article: A. Bertaina, A. Zorzoli, A. Petretto, G. Barbarito, E. Inglese, P. Merli, C. Lavarello, L. P. Brescia, B. De Angelis, G. Tripodi, L. Moretta, F. Locatelli & I. Airoldi (2017) Zoledronic acid boosts γδ T-cell activity in children receiving $\alpha\beta^+$ T and $CD19^+$ cell-depleted grafts from an HLA-haplo-identical donor, OncoImmunology, 6:2, e1216291, DOI: 10.1080/2162402X.2016.1216291

To link to this article: https://doi.org/10.1080/2162402X.2016.1216291

ONCOIMMUNOLOGY
2017, VOL. 6, NO. 2, e1216291 (15 pages)
http://dx.doi.org/10.1080/2162402X.2016.1216291

Taylor & Francis
Taylor & Francis Group

ORIGINAL RESEARCH

Zoledronic acid boosts $\gamma\delta$ T-cell activity in children receiving $\alpha\beta^+$ T and CD19$^+$ cell-depleted grafts from an HLA-haplo-identical donor

A. Bertaina[a,*], A. Zorzoli[b,*], A. Petretto[c,*], G. Barbarito[b], E. Inglese[c], P. Merli[a], C. Lavarello[c], L. P. Brescia[a], B. De Angelis[a], G. Tripodi[d], L. Moretta[e], F. Locatelli[a,f,#], and I. Airoldi[b,#]

[a]Department of Pediatric Hematology and Oncology, IRCCS Ospedale Bambino Gesù, Rome, Italy; [b]Laboratorio di Oncologia, Istituto Giannina Gaslini, Genova, Italy; [c]Core Facilities, Istituto Giannina Gaslini, Genova, Italy; [d]Dipartimento Ricerca Traslazionale, Medicina di Laboratorio, Diagnostica e Servizi, Istituto Giannina Gaslini, Genova, Italy; [e]Area di Ricerca Immunologica, IRCCS Ospedale Bambino Gesù, Rome, Italy; [f]Department of Pediatric Science, Università di Pavia, Pavia, Italy

ABSTRACT

We demonstrated that $\gamma\delta$ T cells of patients given HLA-haploidentical HSCT after removal of $\alpha\beta^+$ T cells and CD19$^+$ B cells are endowed with the capacity of killing leukemia cells after *ex vivo* treatment with zoledronic acid (ZOL). Thus, we tested the hypothesis that infusion of ZOL in patients receiving this type of graft may enhance $\gamma\delta$ T-cell cytotoxic activity against leukemia cells. ZOL was infused every 28 d in 43 patients; most were treated at least twice. $\gamma\delta$ T cells before and after ZOL treatments were studied in 33 of these 43 patients, till at least 7 mo after HSCT by high-resolution mass spectrometry, flow-cytometry, and degranulation assay. An induction of Vδ2-cell differentiation, paralleled by increased cytotoxicity of both Vδ1 and Vδ2 cells against primary leukemia blasts was associated with ZOL treatment. Cytotoxic activity was further increased in Vδ2 cells, but not in Vδ1 lymphocytes in those patients given more than one treatment. Proteomic analysis of $\gamma\delta$ T cells purified from patients showed upregulation of proteins involved in activation processes and immune response, paralleled by downregulation of proteins involved in proliferation. Moreover, a proteomic signature was identified for each ZOL treatment. Patients given three or more ZOL infusions had a better probability of survival in comparison to those given one or two treatments (86% vs. 54%, respectively, $p = 0.008$). Our data indicate that ZOL infusion in pediatric recipients of $\alpha\beta$ T- and B-cell-depleted HLA-haploidentical HSCT promotes $\gamma\delta$ T-cell differentiation and cytotoxicity and may influence the outcome of patients.

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BCP, B-cell precursor; BLM, Bloom syndrome protein; CI, confidence interval; CM, central memory; CMV, cytomegalovirus; E, effector; EM, effector memory; GvHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; IDO, Indoleamine 2,3-dioxygenase; mAbs, monoclonal antibodies; OS, overall survival; PBMC, peripheral blood mononuclear cells; SVM, support vector machine; T, targets; TD, terminally differentiated; TNFAIP8L2/TIPE2, tumor-necrosis-factor-induced protein 8-like 2; ZOL, zoledronic acid

ARTICLE HISTORY
Received 31 May 2016
Revised 10 July 2016
Accepted 18 July 2016

KEYWORDS
$\gamma\delta$ T cells; haematopoietic stem cell transplantation; leukemic patients; proteomic; zoledronic acid

Introduction

Hematopoietic stem cell transplantation (HSCT) from an HLA-haploidentical relative (haplo-HSCT) offers an immediate transplant treatment virtually to any patient in need of an allograft and lacking a suitable matched donor.[1] The use of haplo-HSCT was initially hampered by the risk of graft rejection, graft-versus-host disease (GvHD), and delayed immune reconstitution.[2,3] Intensification of the conditioning regimen, combined with infusion of high numbers of haematopoietic progenitors and with profound *ex vivo* T-cell depletion of the graft, efficiently prevented both graft rejection and GvHD.[2,3] However, delayed immune recovery leading to an increased incidence of opportunistic infections was for many years an obstacle to a wider use of this type of allograft.[4] A promising approach to circumvent such delay is represented by the use of a recently developed method of graft manipulation, based on the selective depletion of $\alpha\beta$ T lymphocytes, and of B cells,[5,6], that allows to transfer to the recipient not only HSC, but also mature donor NK and $\gamma\delta$ T cells, which exert their protective effect against both leukemia cell re-growth and life-threatening infections. Human $\gamma\delta$ T cells orchestrate cellular activities of both innate and adaptive immunity[7-11] and, unlike $\alpha\beta$ T lymphocytes, recognize tumors in a MHC-independent manner and do not cause GvHD.[7,11] These lymphocytes elicit antitumor responses, and have clinical appeal based on their cytotoxicity toward tumor cells and on their ability to present tumor-associated antigens.[12] Among circulating $\gamma\delta$ T cells, there is a major subset expressing Vδ2 chain and a minor subset expressing Vδ1 chain. Both subsets share antitumor properties,[11,13] but Vδ1 cells reside also within epithelial tissues, especially at sites of CMV replication,[14] and may undergo selective expansion in transplanted patients upon

CONTACT I. Airoldi, PhD irmaairoldi@ospedale-gaslini.ge.it Laboratorio di Oncologia, Istituto Giannina Gaslini, Via G. Gaslini 1, 16147 Genova, Italy
Supplemental data for this article can be accessed on the publisher's website.
*These authors equally contributed as first author to this work.
#These senior authors equally contributed as last author.

cytomegalovirus (CMV) reactivation.[8-10,15,16] The Vδ2 population recognizes non-peptide phospho-antigens, may be expanded and activated *ex vivo* and *in vivo* by aminobisphosphonates, such as zoledronic acid (ZOL),[17] thus resulting in an attractive immunotherapeutic tool against cancer.

Current adoptive immunotherapy approaches are limited to the Vδ2 cell subpopulation due to limited expansion of Vδ1 cells to reach numbers sufficient for clinical applications. ZOL infusion resulted in objective clinical responses against both solid and hematologic tumors,[17-20] but was not curative as monotherapy. Vδ1 cells have not yet been infused in clinical trials, but their presence was associated with complete responses observed in patients with B-cell acute lymphoblastic leukemia (ALL) after T-cell-depleted allogeneic HSCT.[21,22]

We recently studied γδ T-cell reconstitution in children after B- and αβ T-cell-depleted haplo-HSCT and demonstrated that these cells exert cytotoxic effects against primary leukemias.[15] Such an activity was strongly potentiated, especially in Vδ2 cells, upon *ex vivo* exposure to ZOL. These data provided a biological rationale for the development of clinical approach based on *in vivo* administration of ZOL in the post-transplantation period, with the aim of improving γδ T-cell cytotoxic capacity against leukemia cells, potentially preventing leukemia relapse. With this background, we have implemented a study investigating the effect on γδ T cells of *in vivo* sequential exposure to ZOL in 43 children receiving a B- and αβ T-cell-depleted haplo-HSCT.

Results

γδ T cells in pediatric recipients of T- and B-cell-depleted haplo-HSCT after ZOL infusion

Flow-cytometry analyses performed on peripheral blood mononuclear cells (PBMC) collected before the first ZOL infusion (3 to 4 weeks after HSCT) showed that circulating T lymphocytes were predominantly of the γδ T-cell lineage (mean 61% of gated $CD3^+$ lymphocytes, range from 34 to 91%). Afterwards, the αβ T-cell population gradually increased (not shown) and the γδ T-cell population decreased over time (Fig. 1A), as already reported for a different cohort of leukemia patients that we previously published,[15] and who had received the same type of graft without being treated with ZOL (controls). Comparative analyses of γδ T cells, Vδ1, and Vδ2 subsets in controls and in ZOL-treated pts, revealed that, 3 mo after HSCT, a significant increase of the percentage of Vδ1 cells (Fig. 1B, left panel), paralleled by a decrease of the percentage of Vδ2 cell subset occurred (Fig. 1B, right panel). Such behavior was observed until month 6, when the percentage of γδ T cells was found to be significantly lower in ZOL-treated patients (pts) than in controls (Fig. 1A). These results suggest that ZOL infusion may influence the differentiation and/or the proliferation of γδ T cells and of their subsets.

We recently described[15] that γδ T cells from recipients of haplo-HSCT express cytotoxic molecules, as well as produce IFNγ. Thus, we tested whether ZOL infusion maintained γδ T cells fully functional to lyse target cells. As shown in Fig. 1C, γδ T cells from recipients of haplo-HSCT treated with ZOL expressed perforin, granzyme-B and, upon calcium ionophore/PMA stimulation, produced IFNγ. Next, we asked whether clinical outcome may be influenced by the percentage of peripheral Vγ9Vδ2 T cells, as observed by others[23] in patients with advanced breast cancer treated with ZOL. To this end, we compared the percentage of peripheral Vγ9Vδ2 cells in patients who had received at least two ZOL infusions and who died with that found in those who were alive at the end of the study. As shown in Fig. 1D, transplanted patients who remained alive showed significantly higher proportion of Vγ9Vδ2 cells (approximately at month 3 after HSCT), suggesting that the persistence of these cells may provide clinical benefit also in leukemia patients receiving haplo-HSCT.

ZOL influences the relative proportion of Vδ1 and Vδ2 subsets in circulating γδ T cells

Next, we evaluated whether the increase in the percentage of Vδ1 cells and the decrease of Vδ2 cells observed over time was dependent exclusively on CMV reactivation, as already reported,[10,15] and/or also on ZOL infusion. Thus, we analyzed the percentage of Vδ1 and Vδ2 populations in patients who either did or did not reactivate CMV, as well as in all patients taken together, irrespectively of CMV reactivation, before and after one or more ZOL infusions. As reported in Fig. 2A, analysis of all patients together revealed that the percentage of Vδ1 cells significantly increased and Vδ2 cells decreased upon the second/third, but not the first ZOL treatment. In particular, the Vδ1 subset became the main circulating γδ T-cell population upon the third ZOL infusion.

Superimposable results were obtained by comparative analysis of patients who did experience CMV reactivation versus those who did not, thus demonstrating that the significant increase of the Vδ1 population was dependent on ZOL exposure and not mainly on virus reactivation (Fig. 2B, white plots). However, in patients experiencing CMV reactivation, the Vδ1 population was expanded and represented the main γδ T-cell subset in both ZOL-treated and untreated patients (Fig. 2B, gray plots and 2C).

ZOL infusion induces differentiation and increases cytotoxicity of Vδ2 cells in vivo

Phenotypic analyses revealed that, before ZOL treatment, 14.5% of Vδ2 lymphocytes were *naïve*, 44.9% were CM, 22.6% EM, and 15.1% TD (Fig. 3A). The percentage of CM Vδ2 cells significantly decreased and that of TD increased starting from the first ZOL administration (Fig. 3A). The *naïve* and EM Vδ2 populations were not affected by ZOL treatment.

Next, we investigated whether Vδ2 cells from ZOL patients were able to lyse target leukemia cells of both myeloid and lymphoid origin, by analyzing CD107a surface expression. Thus, AML or ALL primary blasts, either untreated or treated overnight with ZOL, were co-cultured with PBMC freshly isolated from patients before, after the first, the third, and the fifth ZOL infusion. As shown in Fig. 3B, Vδ2 cells obtained from patients before ZOL treatment and cultured with AML/ALL blasts, expressed CD107a on the cell surface (mean percentage of $CD107^+$ cells in gated Vδ2 subset being 11.56). *In vitro* pre-treatment of blasts with ZOL significantly increased the proportion of $CD107a^+$ cells in gated Vδ2 (mean percentage

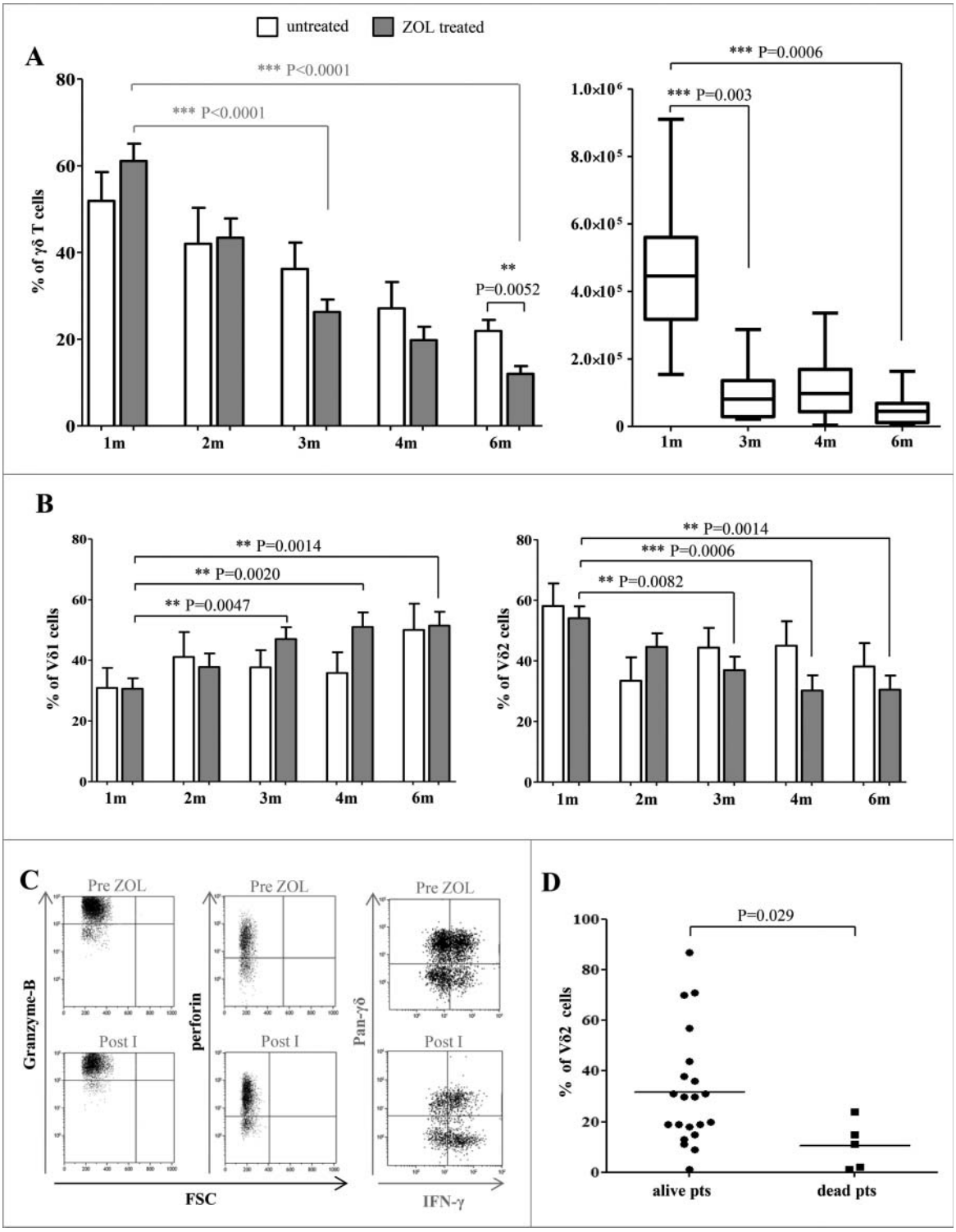

Figure 1. Modulation of $\gamma\delta$ T cells by ZOL infusion. (A) Left panel: Flow cytometry analyses of $\gamma\delta$ (gated in $CD3^+$ T cells) 1, 2, 3, 4, and 6 mo after HSCT. Comparative analyses of proportion of these populations from transplanted patients receiving ZOL infusion (gray bars) and controls (white bars) were performed. Right panel: absolute numbers of $\gamma\delta$ T cells from ZOL-treated pts at 1, 3, 4, and 6 mo after HSCT. (B) Proportion of Vδ1 (left panel) and Vδ2 (right panel) cells (gated in $CD3^+\gamma\delta^+$ T cells) in transplanted patients receiving ZOL infusion (gray bars) and controls (white bars). (C) PBMC collected from haplo-HSCT was stained with perforin-APC or granzyme-B PE or, upon calcium ionophore/PMA stimulation, IFNγ APC mAb. Upper panels show the expression in one representative pt before ZOL infusion (pre ZOL) of granzyme-B (left), perforin (middle) both in gated in $CD3^+/\gamma\delta^+$ T cells, and IFNγ (right) in $\gamma\delta$ cells using an anti TCR$\gamma\delta$ PE-CF594 mAb (gated in $CD3^+$ T lymphocytes). Lower panels show the expression of the same molecules in one representative pt after the first ZOL infusion (post I). (D) Comparative analyses of Vδ2Vγ9 (gated in $CD3^+\gamma\delta^+$ T cells) cells performed, by flow cytometry, in patients receiving two ZOL infusions and that died versus those that were alive at the end of the study.

being 22.57) cells, this suggesting that such $\gamma\delta$ T-cell population exerts cytotoxic functions when the target expresses high levels of phosphoantigens. More importantly, $\gamma\delta$ T cells acquired more activated features upon ZOL infusion, as witnessed by the significant increase of CD107a in Vδ2 cells, from ZOL-treated patients, when challenged with untreated blasts (Fig. 3B).

Noteworthy, Vδ2 cells from patients treated once further increased their cytotoxic activity against leukemia cells when

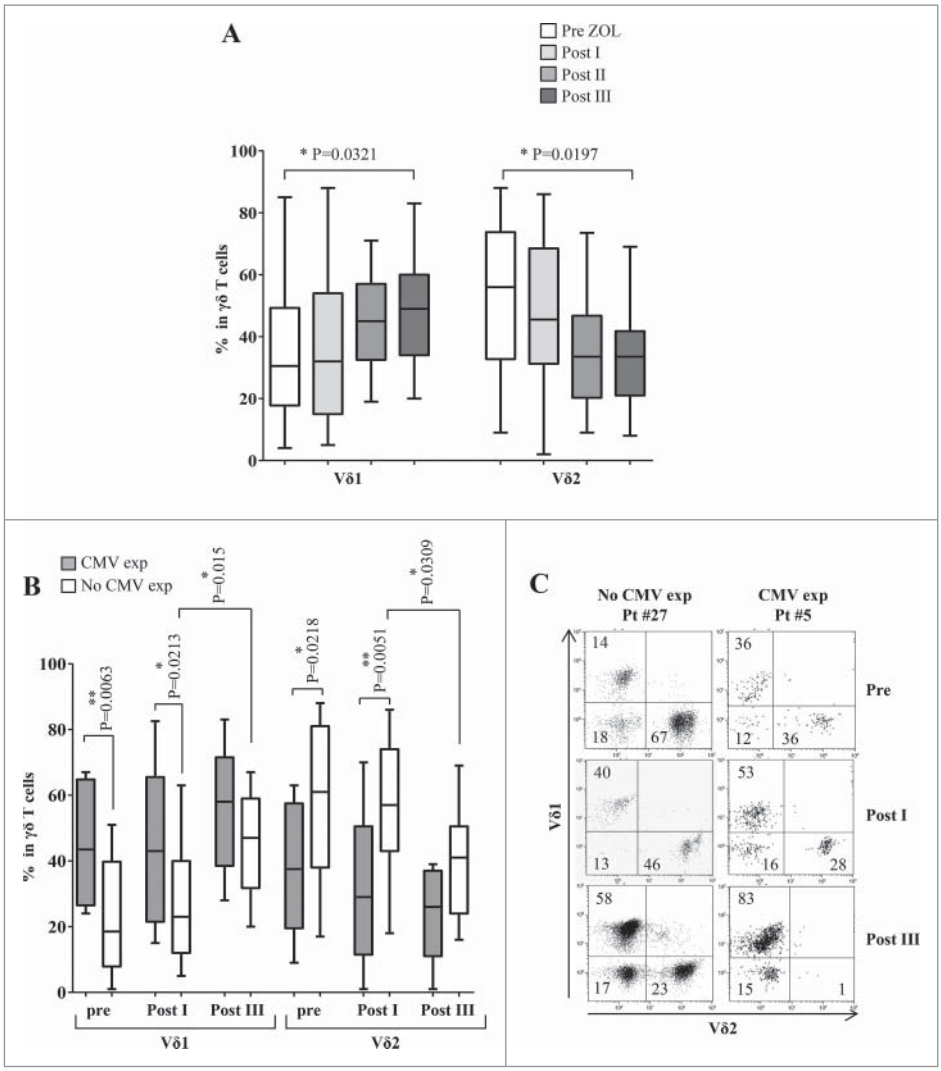

Figure 2. Modulation of $\gamma\delta$ T cell phenotype *in vivo* by ZOL infusion. (A) Flow cytometry analyses of Vδ1 and Vδ2 (gated in $CD3^+\gamma\delta^+$ T cells) before (pre, white plots) and after the first (post I), the second (post II), and the third (post III) ZOL infusion in transplanted patients. Pooled results are shown. Whisker lines represent highest and lowest values, horizontal lines represent median values. (B) Comparative analysis of Vδ1 and Vδ2 T cells was performed in patients who did experience CMV reactivation (gray plots) and in those who did not (white plots). Vδ1 and Vδ2 cells were identified in gated $CD45^+CD3^+\gamma\delta^+$ cells and phenotypically evaluated before and after the first (post I) and the third (post III) ZOL treatment. (C) Percentage of Vδ1 and Vδ2 subsets, in gated $CD45^+CD3^+\gamma\delta^+$ cells, in PBMC from two representative cases is shown.

target cells had been previously treated with ZOL. By contrast, Vδ2 cells from patients treated three times or more with ZOL significantly increased their cytotoxic activity against untreated leukemia cells, especially when compared to Vδ2 cells obtained from patients treated only once (Fig. 3). No significant differences were observed in CD107a surface expression in Vδ2 cells from patients treated 3/5 times and challenged with AML or ALL cells treated with ZOL versus Vδ2 cells cultured with untreated AML or ALL blasts (Figs. 3B and C). Thus, Vδ2 cells from patients receiving repeated infusions of ZOL appeared to be activated and to exert cytotoxic functions irrespectively of the level of phosphoantigens expressed by target cells.

ZOL infusion stimulates Vδ1 cytotoxicity in vivo

As shown in Fig. 3D, starting from the second month after HSCT, a significant decrease, which has been reinforced over time, of the CM subset of Vδ1 cells was observed. This decrease was paralleled by an increase, although not statistically significant, of the TD subset, thus suggesting an induction of differentiation. The Vδ1 population, collected from patients treated once with ZOL, was able to exert cytotoxic functions, when challenged with primary leukemia cells either untreated or treated with ZOL, as demonstrated by the significant upregulation of CD107a surface expression (Figs. 3E and F). Subsequent ZOL infusions were unable to induce additional stimulation of Vδ1 cytotoxic activity (Figs. 3E and F).

Details of patients used for studying the in vivo effects of ZOL on $\gamma\delta$ T cell proteome profile after the first infusion

Proteomic analyses were performed using $\gamma\delta$ T cells purified from PBMC of seven patients (#21, #22, #23, #25, #27, #29, and #32) before (pre) and eight patients (#20, #21, #22, #23, #24, #25, #27, and #32) 20–25 d after the first infusion with ZOL (post I). Quality control of proteome profiles in each sample, based on unsupervised hierarchical cluster, revealed the following: (i) pt#32 pre-treatment and from pt#20 were outliers, probably due to a not efficient sample preparation, and (ii) pt#21 and #27 after ZOL treatment showed a profile very similar to that of untreated

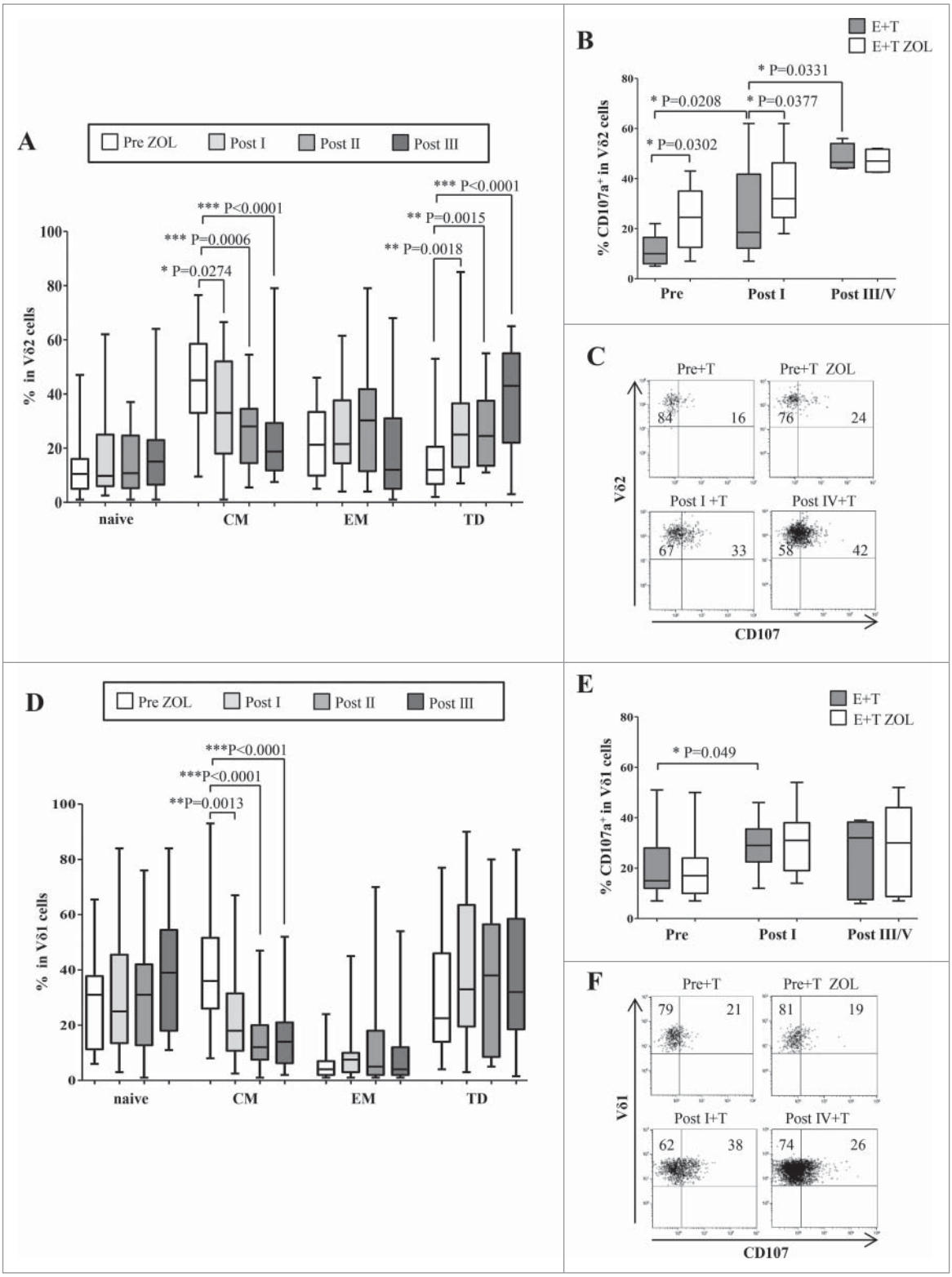

Figure 3. Differentiation and cytotoxicity of Vδ2 and Vδ1 cells in ZOL-treated patients. (A) Percentage of *naïve*, CM, EM, and TD subsets was evaluated, by flow cytometry, in gated $CD3^+\gamma\delta^+V\delta2^+$T cells from PBMC of transplanted patients before (pre), after the first (post I), the second (post II), and the third (post III) ZOL infusion. Pooled results are shown. Whisker lines represent highest and lowest values, horizontal lines represent median values. (B) CD107a expression was investigated in gated $\gamma\delta^+V\delta2^+$ T cells, cultured with ALL or AML blasts untreated (T) or after ZOL-exposure (T+ZOL). Analyses were performed in PBMC from patients before (pre), after the first (post I) and the third or fifth (post III/V) ZOL infusion. (C) One representative experiment using PBMC obtained from patient #1, before or after sequential ZOL infusion, co-cultured with AML blasts untreated (T med) or ZOL-sensitized (T ZOL) is shown. (D) Percentage of *naïve*, CM, EM, and TD subsets was evaluated by flow cytometry in gated $CD3^+\gamma\delta^+V\delta1^+$T cells from PBMC of transplanted patients before (pre), after the first (post I), the second (post II), and the third (post III) ZOL infusion. Pooled results are shown. Whisker lines represent highest and lowest values, horizontal lines represent median values. (E) CD107a expression was investigated by flow cytometry in gated $\gamma\delta^+V\delta1^+$ T cells, cultured with ALL or AML blasts untreated (T) or ZOL-sensitized (T+ZOL). Analyses were performed in PBMC from patients before (pre), after the first (post I) and the third or fifth (post III/V) ZOL infusion. (F) One representative experiment using PBMC obtained from patient #1, before or after sequential ZOL infusion, co-cultured with AML blasts untreated (T med) or ZOL-sensitized (T ZOL) is shown.

patients. On the basis of such considerations, these samples were not included in subsequent statistical analysis.

Thus, only pre-ZOL samples from pt#21 and #27 could be evaluated by T-test that was subsequently used to understand whether ZOL was effective on γδ T cells, irrespectively of the patient analyzed or of the time of treatment. Although the proteomic profile of γδ T cells from pt #27 was not significantly modulated by the first ZOL infusion, significant proteomic changes were observed after the second and the third ZOL treatment (see Figs. 5 and 6). The proteomic profile of γδ T cells from pt#29 was similar to that observed in all the other patients before ZOL infusion, thus it was not included in T-Test analysis, but analyzed for the evolution of the proteotype (see below and Fig. 6).

In vivo effects of ZOL on γδ T cell proteome profile after the first infusion

We investigated whether the first ZOL infusion could modulate the protein profile in γδ T cells isolated from the PB of transplanted patients. MaxQuant analysis identified 4,722 proteins, of which 3,895 were quantified. Applying a T-Test (FDR<0.01 S0>0.5), we found 377 proteins significantly modulated (Fig. 4A), of which 149 were downregulated (Cluster 1) and 228 were upregulated after the first ZOL infusion (Cluster 2), see Heat Map (Figs. 4B and C). Using Fisher's exact test on the GOBP of the two protein clusters, different proteins involved in nucleic acid metabolic process, RNA and mRNA processing, transcription, regulation of gene expression, and chromatin remodeling resulted to be downregulated in cluster 1, compared to cluster 2. By contrast, proteins involved in platelet activation, response to stimulus, cell activation, defense response, regulation of secretion, immune response, and toll-like receptor 2 signaling pathway were found to be upregulated upon ZOL infusion (Figs. 4C and D). These effects are uniquely due to ZOL treatment and not dependent on time. Indeed, patients were treated at different days from transplantation (pt#21 at +79, pt#22 at +52, pt#23 at +34, pt#25 at+26, and pt#27 at +90, Fig. 4B), thus the point "post I" corresponded to day +79 from HSCT in pt#22, +60 in pt#23 and #24, +49 in pt#25, and +55 in pt#32.

Proteomic analysis of γδ T cells from transplanted children after sequential ZOL treatments

The above results led us to investigate whether sequential ZOL infusion may enhance such effects. For this purpose, purified γδ T cells obtained 20–25 d after the second (post II, pt#22, #24, #25, and #27) and the third (post III, pt#22, #24, #25, #27, and #29) ZOL infusion were analyzed for the evolution of proteotype. The B Significance Test (Benjamini–Hochberg FDR <0.05) identified 138 proteins, out of the 4,455 quantified, that were significantly modulated in γδ T cells after the third ZOL infusion compared to those found before treatment. GO enrichment analysis revealed an induction in different activation pathways, including platelet activation and degranulation, secretion and focal adhesion, leukocyte chemotaxis, and migration (Fig. 5A). More in detail, subsequent ZOL infusions into the same patient caused a gradual increase of the intensity, and therefore of abundance, of the same proteins already found upregulated after the first infusion (red plots in Fig. 5B). Results of the analyses of γδ T cells from pt#27 are shown in the quantitative profile plots (left panel) and in the Heat Map (right panel) of Fig. 5C. Similar results were obtained from pt#25 (Fig. S1A) and pt#22 (Fig. S1B) and from the remaining two patients (not shown). The common patterns of pathways modulated by ZOL infusions in each individual patient are shown in Fig. S1C.

Proteomic analysis identifies a signature for each individual ZOL infusion

Finally, we used support vector machine (SVM) classification algorithm, and obtained a signature that recognizes a specific protein pattern for each treatment. The advancements in mass-spectrometry have enabled the routine identification and quantification of thousands of proteins. We describe an *in vivo* clinical proteomic dataset that offers the opportunity to classify a γδ T-cell proteotype specific for each treatment. Due to the biological variability among patients, we used a machine learning and statistical method that integrates SVM with various feature selection methods for the successful classification of clinical proteomics samples. We analyzed the proteome profiles of transplanted patients and, based on feature ranking, we selected 57 proteins that discriminate the result of each individual ZOL treatment (Fig. 6). Moreover, the 57 proteins selected from the Learning Machine were further sorted by immunological relevance querying the GO Immunosystem database. From these annotations, we built a functional network, where nodes represent the biological functions, and the interactions are the proteins common to each node (Fig. S2). With this approach, we selected 15 proteins that may be functionally relevant, due to their involvement in differentiation processes or regulation of immune response, including the following: (i) tumor-necrosis-factor-induced protein 8-like 2 (TNFAIP8L2/TIPE2), associated with the first treatment; (ii) Bloom syndrome protein (BLM) and Indoleamine 2,3-dioxygenase (IDO), selectively induced upon the second ZOL infusion; and (iii) DOCK1 and FcεR1γ specific of the third infusion. Thus, these proteins may be considered markers of the effectiveness of ZOL infusion. Noteworthy, none of these proteins has been previously associated with γδ T-cell functions; thus, the investigation on their role deserves further studies.

Outcome of patients enrolled in the study

In the whole cohort of 43 patients given ZOL, the probability of overall survival (OS) was 77.9% (95% Confidence Interval, CI, 61.6–88), whereas the cumulative incidence of acute and chronic GVHD were 18.6% (95% CI 6.1–29.4) and 5% (95% CI 0–11.5), respectively. Neither the probability of OS nor the cumulative incidences of both acute and chronic GVHD differed in the population of 33 children selected for the biological study (data not shown). Moreover, the incidence of acute and chronic GVHD in these 43 children was comparable to that of 80 patients with acute leukemia in morphological remission treated with the same transplant approach, without being treated with ZOL [acute GVHD 30% (95% CI 21–42), chronic

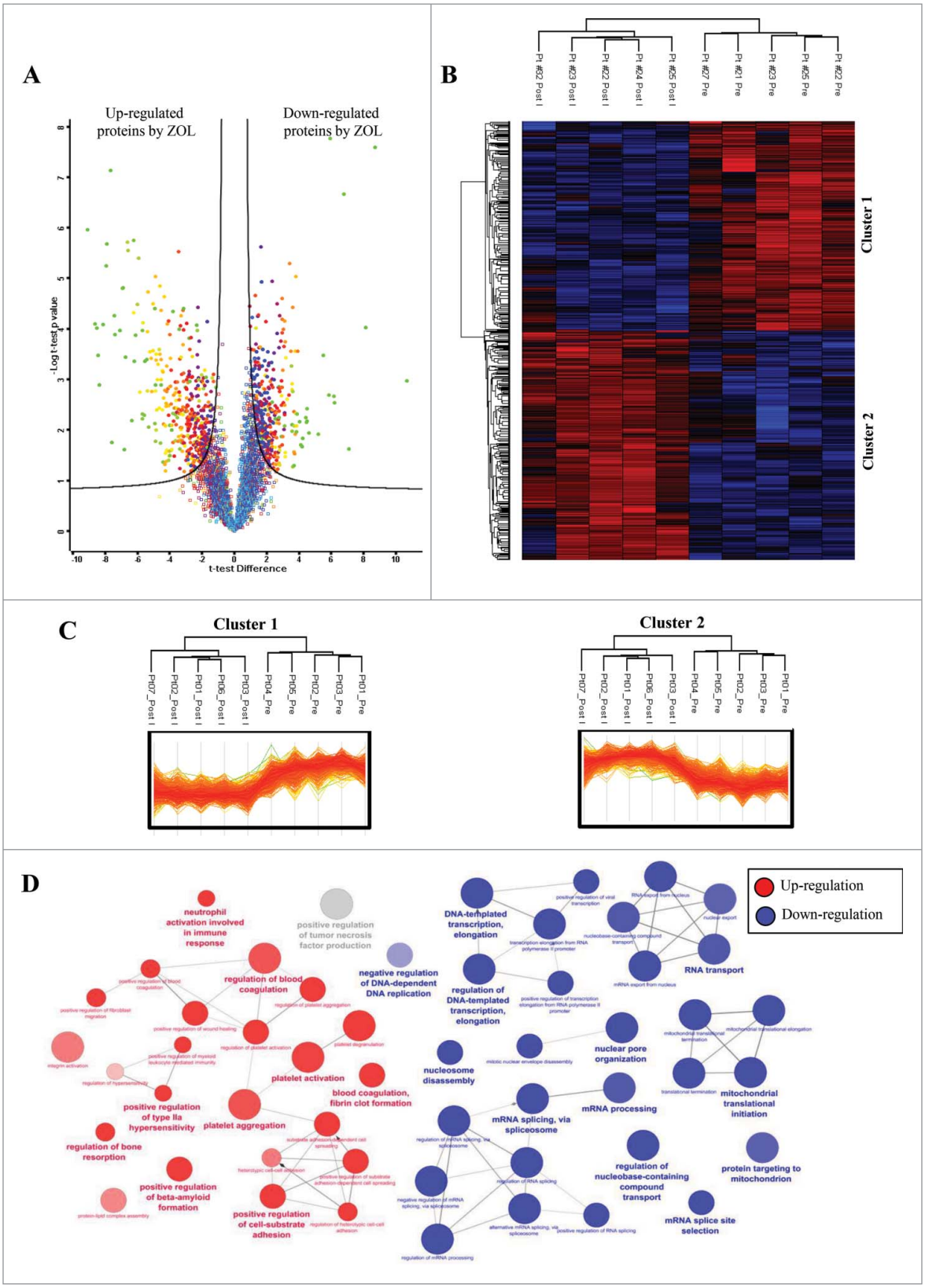

Figure 4. Proteomic analysis in $\gamma\delta$ T cells circulating in transplanted patients before and after the first ZOL infusion. (A) Volcano plot was used to represent graphically the results of the T-tests for differential expression of proteins identified in $\gamma\delta$ T cells before and after the first ZOL treatment. The log2 fold change for each protein is plotted against the $-$log 10 of the *p*-value. The polygon (FDR$<$0.05, S0$>$ 0.5) splits the significant proteins from those that are not modulated. The $\gamma\delta$ T-cell proteins downregulated (left) and upregulated (right) in patients after ZOL treatment, are shown. (B) Heat Map of proteins significantly modulated by the first ZOL infusion in each patient. Comparative analysis was performed between proteins identified in $\gamma\delta$ T cells from patients before and after ZOL infusion. The color code represents the density of points in the corresponding colored region. In red are shown the upregulated proteins and in blue those downregulated. (C) The hierarchical clustering of significantly modulated proteins by the first ZOL infusion in transplanted patients is shown. Cluster 1 (149 proteins) represents significantly downregulated, and Cluster 2 (228 proteins) significantly upregulated proteins in $\gamma\delta$ T cells upon *in vivo* ZOL infusion. (D) The functionally grouped network that visualizes the non-redundant biological terms of GOMF and KEGG derived by a Fischer's Exact Test, on protein significantly modulated in $\gamma\delta$ T cells circulating in transplanted patients after the first ZOL treatment. Red circles represent the upregulated pathways, whereas the blue circles those downregulated.

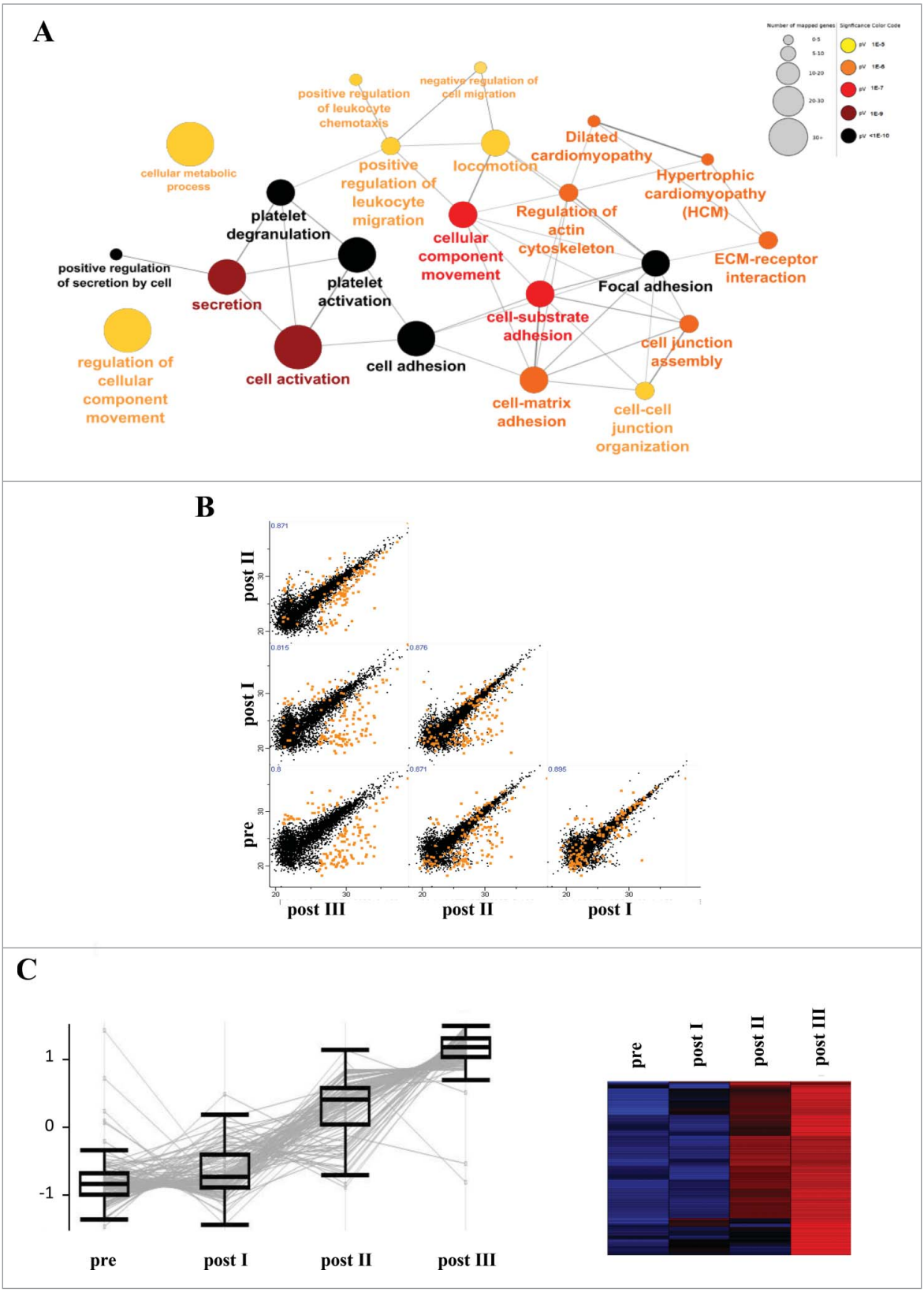

Figure 5. Proteomic analysis of $\gamma\delta$ T cells from transplanted patients after sequential ZOL infusion. (A). Functionally grouped network, visualizing the non-redundant biological terms of GOMF and KEGG derived by a Fischer's Exact Test on proteins significantly regulated, is shown. Analysis was performed using purified $\gamma\delta$ T cells from pt #27 before treatment versus $\gamma\delta$ T cells collected from the same patient after the first, the second, and the third ZOL infusion. (B) Dynamic range of proteomes in pt #27, based on raw protein intensity, is shown. Red dots are the significant features, determined by a B significance test, that increase intensity after sequential treatment (Post I, Post II, Post III) with ZOL. (C) Quantitative Profile Plot (left) and Heat Map (right) of proteins significantly modulated by consecutive treatments with ZOL in $\gamma\delta$ T cells from pt #27. The color code in the Heat Map represents the density of points in the corresponding colored region. In red are shown the upregulated proteins and in blue those downregulated.

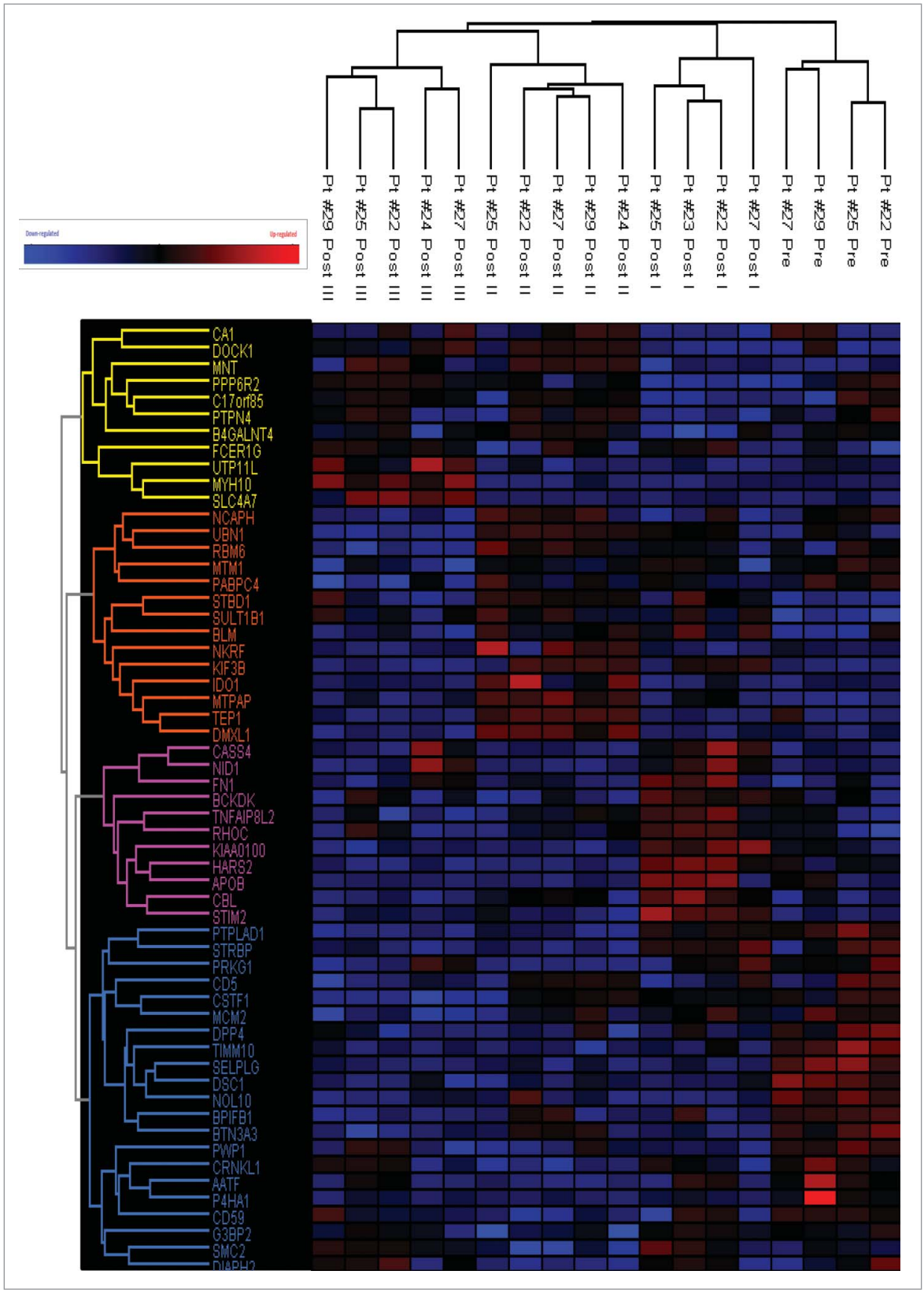

Figure 6. Proteomic signature distinctive of the first, the second, and the third infusion of ZOL in transplanted patients. Heat Map of proteins identified in $\gamma\delta$ T cells distinctively modulated by the first (post I) or the second (post II) or the third (post III) ZOL infusion. The color code represents the density of points in the corresponding colored region. In red are the upregulated proteins and in blue those downregulated. Machine learning algorithms were used to obtain a predictive signature in $\gamma\delta$ T cells purified from patient PBMC to distinguish different proteins modulated by the first, the second, or the third ZOL treatment. Data were filtered to retain only proteins with numerical values in at least four of five samples. Missing values are replaced by an imputed random value that creates a normal distribution with a downshift of 1.8 standard deviations and a width of 0.3 of the original distribution. SVM algorithm was used for classification with polynomial Kernel and RFE-SVM based feature ranking. The number of features was chosen based on the lowest error percentage. Hierarchical clustering was done after z-score normalization of the proteins, and was based on Euclidean distances between averages.

GVHD 5.5% (95% CI 2–14)]. Patients given three or more infusions had a lower incidence of both acute and chronic GvHD than patients given one or two infusions (data not shown); there was also a trend toward a lower incidence of CMV infection in patients given more infusions, but the difference was not statistically significant (data not shown). Notably, when we stratified the outcome of patients according to the number of ZOL doses infused, we found that the 32 children given three or more ZOL infusions had a better probability of OS than the 11 children receiving either one or two doses of the drug [86% (95% CI 66.3–94.6) vs. 54.5% (95% CI 22.9–78), respectively; $p = 0.008$]. These findings suggest that ZOL does not increase the risk of either acute or chronic GVHD and that three or more doses of the drug may improve patients' outcome.

Discussion

$\gamma\delta$ T lymphocytes are a peculiar subset of T cells that contribute to host immune response, uniquely combining conventional adaptive features with rapid, innate-like responses. $\gamma\delta$ T cells: (i) recognize tumor antigens in MHC-independent manner; (ii) have endogenous cytotoxicity; (iii) produce cytokines useful to mount antitumor and antiviral responses[11]; (iv) may be *ex vivo* expanded and activated with ZOL; and (v) may develop immunological memory.[24,25] These features render $\gamma\delta$ T cells an appealing immunological population to fight cancer cell regrowth and viral infections, both issues representing major problems in patients given HSCT. ZOL has been approved by Food and Drug Administration for the treatment of metastatic bone involvement by hematopoietic tumors, such as multiple myeloma,[26] and by solid tumors, including breast and prostate cancers.[27,28] In children, a phase I study of ZOL in recurrent/refractory neuroblastoma showed clinical and biological response, with mild toxicity.[29]

With this background, we have investigated whether ZOL infusion in pediatric recipients of $\alpha\beta$ T-and B-cell-depleted haplo-HSCT may influence both functional behavior of peripheral $\gamma\delta$ T cells and patients' outcomes. Here, we report the effects exerted *in vivo* by ZOL on $\gamma\delta$ T cells, using classical phenotypical and functional assays, synergistically integrated with innovative proteomic tools of sample preparation,[30] analytical conditions,[31,32] high-resolution mass spectrometry,[33] statistical,[34-36] and network analysis.[37] These novel proteomic approaches have been here applied to clinical studies and high-resolution mass spectrometry, based on orbitrap technology, is characterized by high reproducibility, sensibility, and specificity. The *in vivo* evolution of $\gamma\delta$ T-cell proteotype mediated by ZOL was characterized by upregulation of proteins involved in activation pathways and by the downregulation of proteins of proliferative pathways. Such effect, already evident after the first ZOL infusion, but further boosted by the subsequent infusions, mirrored the phenotypic changes observed through flow cytometry in both Vδ1 and Vδ2 subsets. ZOL influenced, unexpectedly, the phenotype and function not only of Vδ2 cells, which selectively recognize phosphoantigens, but also of the Vδ1 population. In particular, the first treatment with ZOL induced the differentiation of Vδ2 cells, which showed a maturation trend, moving from a CM to an EM/TD phenotype. This maturation was associated with immediate effector functions,[38,39] a result supported by our functional experiments highlighting a boost of Vδ2 cell cytotoxicity against primary leukemia cell blasts, irrespectively of their phosphoantigen expression. The anti-proliferative effect of ZOL on total $\gamma\delta$ T cells, identified by proteomic studies, was reflected by the decrease of the Vδ2 population starting from month 3 after HSCT. By contrast, the percentage of Vδ1 subset increased over time upon ZOL infusion, irrespectively of CMV reactivation. The percentage of Vδ2 cells was found to be higher in transplanted patients that were alive at the end of the study, compared to that observed in patients that died, mainly due to disease recurrence/progression (see also Table 1 for further details). This finding is in accordance with previous observations showing that high numbers of circulating mature and cytotoxic Vγ9Vδ2 cells induced by aminobisphosphonates in patients with malignancies were associated with good prognosis.[17,19]

Phenotypic and functional studies delineated a route of activation in $\gamma\delta$ T cells upon sequential ZOL treatment, which was unambiguously revealed by a specific proteomic signature. Actually, using a learning-machine statistical software, we identified 15 proteins selectively involved in immunological functions, which were further upregulated in $\gamma\delta$ T cells upon each individual ZOL infusion. To date, most of them have not been associated with $\gamma\delta$ T-cell functions and deserve future, specific investigations. Nonetheless, induction of TNFAIP8L2/TIPE2 and BLM captured our attention, since their presence may be relevant in the setting of HSCT. TIPE2 is a cytoplasmic protein predominantly expressed in immune cells and especially in T lymphocytes; abnormal expression of TIPE is implicated in systemic autoimmunity,[40] diabetic nephropathy,[41] and hepatitis B.[42] In addition, TIPE-knockout mice developed a severe colitis, with enhanced leukocyte infiltration, bacterial invasion, and inflammatory cytokine in the colon.[43,44]

A key protein that signed the proteotype of $\gamma\delta$ T cells in transplanted patients treated twice with ZOL is BLM, important in development, maintenance, and function of $\alpha\beta$ T lymphocytes.[45] Mutations of the BLM gene are responsible for Bloom Syndrome, a disorder characterized by immunodeficiency and propensity to develop cancer. The essential role of BLM in early $\alpha\beta$ T-cell differentiation was evidenced by the impairment of T-cell differentiation, proliferation, and response to antigens in *Blm*-deficient mice. It was recently reported that a minor subset of peripheral Vδ1 cells that express $CD4^+$ in association with stemness/progenitor markers may transdifferentiate into $\alpha\beta$ T cells.[46] This demonstration, in addition with our finding that ZOL increased the Vδ1 percentage and induced BLM in $\gamma\delta$ T cells, lets envisage a peculiar scenario in which ZOL may induce *in vivo* a "reservoir" for the development of $\alpha\beta$ T cells. Although detailed studies are needed to validate such a hypothesis, this feature could be of particular relevance in the transplant setting.

In conclusion, we demonstrated that ZOL infusion in patients receiving haplo-HSCT depleted of $\alpha\beta$ T and CD19 B lymphocytes was safe and, once repeated three or more times, effective protecting patients from GvHD occurrence and improving OS. ZOL treatment caused multifunctional beneficial effects on $\gamma\delta$ T cells, our results suggesting new proteomic keys to test the responsiveness of patients to this treatment.

Table 1. Patient characteristics.

Patient # (gender)	Age (years)	Original disease and status at haplo-HSCT	Duration of study monitoring (days from HSCT)	CMV reactivation	Status at last follow-up (causes of death)	ZOL treatment
Pt #1 (M)	8	T-ALL, CR2	339	−	alive	5
Pt #2 (M)	14	T-ALL, CR1	224	+	alive	1
Pt #3 (M)	6	BCP-ALL, CR1	245	−	alive	6
Pt #4 (M)	11	BCP-ALL, CR3	182	+	alive after relapse	3
Pt #5 (F)	13	BCP-ALL, CR1	195	+	alive	4
Pt #6 (F)	6	AML, CR1	93	+	dead (disease progression)	2
Pt #7 (F)	11	BCP-ALL, CR2	208	−	alive	4
Pt #8 (M)	8	BCP-ALL, CR2	248	−	dead (disease progression)	3
Pt #9 (M)°	11	Ph+-ALL, CR2	139	+	dead (TRM, sepsis)	2
Pt #10 (M)	6	BCP-ALL, CR2	225	−	alive	3
Pt #11 (M)	15	AML, CR2	457	−	alive	3
Pt #12 (M)	19	BCP-ALL, CR2	352	−	alive	4
Pt #13 (M)	18	T-ALL, CR1	244	−	alive	1
Pt #14 (M)	18	BCP-ALL, CR2	302	−	alive after relapse	3
Pt #15 (M)°	9	BCP-ALL, CR3	288	+	dead (disease progression)	2
Pt #16 (M)	15	T-ALL, CR2	278	−	dead (disease progression)	3
Pt #17 (M)	14	AML, CR1	243	+	alive	3
Pt #18 (M)	6	T-ALL, active disease	134	+	dead (disease progression)	2
Pt #19 (F)	14	AML, CR2	98	−	dead (disease progression)	2
Pt#20 (M)	15	AML, CR1	240	−	alive	3
Pt#21 (F)	12	BCP-ALL, CR2	226	+	alive	1
Pt#22 (M)	5	BCP-ALL, CR2	162	+	alive	3
Pt#23 (M)	13	Ph+ BCP ALL, CR2	270	−	alive	3
Pt#24 (M)	6	BCP-ALL, CR2	271	−	alive	4
Pt#25 (M)	4	BCP-ALL, CR2	269	−	alive after relapse	4
Pt#26 (M)	10	T-ALL, CR2	138	+	alive	2
Pt#27 (M)	1	AML, CR1	183	−	alive	3
Pt#28 (M)	3	AML, CR2	111	−	alive	4
Pt#29 (M)	15	AML, CR2	189	−	alive	4
Pt#30 (M)	1	AML, CR1	233	−	alive	3
Pt #31 (F)	12	AML, CR1	70	+	alive	3
Pt #32 (M)	2	AML, CR1	112	−	dead (disease progression)	3
Pt #33 (M)	9	BCP-ALL, CR2	185	−	alive	3

Pt = patient; BCP-ALL = B-cell precursor acute lymphoblastic leukemia; T-ALL = T-cell acute lymphoblastic leukemia; AML = acute myeloid leukemia; Ph+ = Philadelphia positive, CR = complete remission; m = male; f = female; HSCT = hematopoietic stem cell transplantation; TRM = transplantation-related mortality; CMV = cytomegalovirus; °these patients had previously received an allograft from an unrelated donor.

Both the clinical and biological results suggesting a benefit for patients treated with ZOL have to be confirmed in a prospective, randomized clinical trial.

Patients and methods

Patients

Forty-three children, 30 with ALL and 13 with acute myeloid leukemia, given allogeneic HSCT from an HLA-partially matched family donor after TCR$\alpha\beta$/CD19 negative selection (ClinicalTrials.gov Identifier: NCT01810120) in morphological complete remission, or with active disease or after having relapsed following a previous HSCT, were given one or more doses of ZOL to optimize $\gamma\delta$ T-cell function/recovery. We investigated the influence of ZOL on $\gamma\delta$ T-cell phenotype, function, and proteomic profile in 33 out of these 43 pts.

Clinical features of patients included in the biological study are reported in Table 1, while the composition of the graft infused in these patients is reported in Table 2. *Ex vivo* assays of immune-cell phenotype were routinely performed at least till month 7 after haplo-HSCT using 3–4 mL of PB. Patients were censored at time of relapse. Samples were collected weekly until hospital discharge and monthly during routine follow-up visits to the outpatient clinic. Among the patients included in this study, 36% (12/33) experienced CMV reactivation.

Treatment schedule

Patients receiving haplo-HSCT were treated with intravenous infusion of ZOL (Zometa from Novartis, 0.05 mg/kg/dose, maximum dose 4 mg), according to a specific protocol approved by the Ethics Committee of Bambino Gesù Children's Hospital. Treatment started at day +28/+35 in 24 pts (56%), at day +41/+60 after HSCT in 7 pts (16%), and at day +71/+79 in 12 pts (28%), these being time points at which all children had already obtained engraftment of donor hematopoiesis and the majority of lymphocytes in PB were represented by $\gamma\delta$ T cells. Concerning patients included in the biological study, treatment started at day +28/+35 in 24 pts (72.7%), at day +74/+79 in 5 pts (15.15%), and at day +52/+60 after HSCT in 4 pts (12.12%). In the absence of any relevant side effect and whenever possible, treatment was repeated every 28 d till month 7. $\gamma\delta$ T cells from PBMC of patients receiving ZOL were phenotypically and functionally studied at day +18/+25 from each treatment.

Antibodies and intracellular staining

The following monoclonal antibodies (mAbs) from BD Biosciences were used: PE-Cy7- (clone SK7), PE-, APC- or PE-CF594-conjugated (clone UCHT1) anti-CD3; FITC-, PE-Cy7- anti-CD45; FITC-conjugated anti-TCR $\alpha\beta$ (clone B3);

Table 2. Graft composition.

Patients	CD34$^+$/Kg (10^6)	TCR-$\alpha\beta$/Kg (10^6)	TCR-$\gamma\delta$/Kg (10^6)	CD56$^+$16$^+$/Kg (10^6)	CD20$^+$/Kg (10^6)
Pt#1	17.4	0.050	9.90	43.82	0.092
Pt#2	11.96	0.017	3.70	13.40	0.016
Pt#3	26.03	0.025	9.30	60.20	0.17
Pt#4	17	0.048	9.60	40	0.07
Pt#5	13.96	0.070	7.56	7.87	0.018
Pt#6	13.80	0.002	8.6	66.70	0.027
Pt#7	12.87	0.071	3.54	10	0.07
Pt#8	15.92	0.080	11.40	16	0.13
Pt#9	12	0.032	11	18	0.004
Pt#10	18.09	0.069	13.45	44.70	0.13
Pt#11	13.09	0.044	15.01	6.85	0.017
Pt#12	12.17	0.028	6.70	12.36	0.18
Pt#13	9.33	0.045	21.75	15.50	0.9
Pt#14	13.25	0.048	8.3	23	0.003
Pt#15	12.8	0.021	9.5	19.6	0.014
Pt#16	15.3	0.027	6.69	24.50	0.053
Pt#17	9.8	0.080	8.40	14	0.031
Pt#18	40.44	0.040	5.45	21	0.01
Pt#19	9.90	0.040	3.70	21.57	0.047
Pt#20	13.34	0.039	6	31.20	0.003
Pt#21	9.78	0.051	15.34	3.84	0.017
Pt#22	12.21	0.027	14.88	51.67	0.005
Pt#23	14.3	0.064	7.06	30.94	0.025
Pt#24	16.7	0.099	17.80	60.70	0.042
Pt#25	20.14	0.078	27.90	57.60	0.022
Pt#26	15.53	0.076	4.71	12.77	0.055
Pt#27	24.4	0.096	37.80	39	0.07
Pt#28	19.6	0.040	17	78.20	0.023
Pt#29	12.2	0.054	7.2	14.1	0.015
Pt#30	18.87	0.010	15.85	146.10	0.025
Pt#31	19.7	0.050	7.9	72	0.057
Pt#32	22	0.045	12	32	0.011
Pt#33	20.30	0.047	7.4	64.17	0.007

APC-, PE-CF594- (clone B1) or PE-conjugated (clone 11F2 or B1) anti-TCR $\gamma\delta$; PE-conjugated anti-Vδ2 (clone B6), APC-conjugated anti-Vγ9 (clone B3); APC-conjugated anti-CD45RO (clone UCHL1); PE-Cy7-conjugated anti-CD27 (clone M-T271); APC-conjugated (clone H4A3) anti-CD107a. FITC-conjugated anti-Vδ1 (clone TS8.2) was from Thermo Scientific. APC-conjugated (clone B27) anti-IFNγ was from BD Biosciences, PE-Cy7-conjugated anti-IFNγ (clone 4S.B3), PE-conjugated anti-granzyme B (clone GB11), PE- or APC-conjugated anti-perforin (clone dG9) were from eBioscience. IFNγ expression was assessed by culturing cells for 3 h in the presence of calcium ionophore (250 ng/mL, Sigma-Aldrich), PMA (20 ng/mL; Sigma-Aldrich) and brefeldin A (5 μg/mL; Sigma-Aldrich). IFNγ, perforin and granzyme B intracellular expression was performed on cells labeled with specific surface markers, fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences), and subsequently incubated with specific mAb.

Phenotype of circulating $\gamma\delta$ T cells

PBMC were enriched by Ficoll-Hypaque (Sigma Aldrich) density gradient centrifugation. We acquired at least 10^5 events of total cells on Gallios® flow-cytometer (Beckman Coulter), which were analyzed using Kaluza® software analysis (Beckman Coulter). Different combinations of monoclonal antibodies allowed identifying main $\gamma\delta$ T-cell subsets: *naïve* (identified as CD45RO$^-$CD27$^+$), CM (CD45RO$^+$CD27$^+$ cells), EM (CD45RO$^+$CD27$^-$), and TD (CD45RO$^-$CD27$^-$) Vδ1 and Vδ2 cells. Percentage of $\gamma\delta$ T-cell subsets were evaluated in gated CD3$^+$/Vδ1$^+$ or CD3$^+$/Vδ2$^+$ lymphocytes.

Degranulation assay

Degranulation assay was performed by co-culturing 10^5 effectors (E) and 10^5 target (T) cells with 3 μL anti-CD107a antibody in 96 V-bottom plates for 3 h at 37°C.

Effectors (E) were PBMC freshly obtained from patients (#1, #2, #3, #4, #5, #6, #9, #12, #14, #15, #17, #18, #19, and #20) before and after 15–20 d from ZOL treatment. Targets (T) were primary AML (n = 3), T-ALL (n = 3), and B-cell precursor (BCP)-ALL (n = 5) blasts cultured overnight with either 20 μM ZOL or medium.

Thereafter, cells were collected, washed in PBS, and stained with anti-CD3, -pan $\gamma\delta$, -Vδ1, -Vδ2, and CD107a analyzed in gated Vδ1 or Vδ2 $\gamma\delta$ T cells, by flow cytometry. At least 1.5 × 10^5 events were acquired.

Evaluation of the protein expression profile in circulating $\gamma\delta$ T cells before and after treatment with ZOL

$\gamma\delta$ T-cell samples were analyzed before (pt#21, #22, #23, #25, #27, #29, and #32) and 20–25 d after the first (pt#20, #21, #22, #23, #24, #25, #27, and #32), the second (#22, #24, #25, and #27), and the third (pt#22, #24, #25, #27, and #29) ZOL treatment. We used 3–4 × 10^5 $\gamma\delta$ T cells, with exception for pt#20, #21, and #32 pre, #24 post II, and post III, #27 and #29 post III infusion from which 1–2.4 × 10^5 $\gamma\delta$ T cells were

purified. Cells were lysed and the extracted proteins subjected to proteolysis employing an in-StageTip method.[30] Samples were analyzed by reversed-phase liquid chromatography coupled to mass spectrometry (LC-MS), in which selected peptides were fragmented by tandem mass spectrometry (MS/MS). Proper statistical data analysis was performed (see Supplemental Materials). We used both machine learning algorithms to classify patients and feature selection algorithms to extract predictive protein signatures for each ZOL treatment.

Statistics

Statistical analysis, with exception for proteomic studies, was performed using GraphPad Prism 5 (Software Inc.). Data distributions were compared using either the *t* test, or the Mann–Whitney or Wilcoxon rank test, whichever appropriate. All statistical tests were two-tailed. Probability of OS was calculated according to the Kaplan and Meier method.[47] Acute and chronic GvHD were evaluated as cumulative incidence curves in order to adjust the estimates for competing risks (i.e., graft rejection, death in remission).[48,49] All results were expressed as probability or cumulative incidence (%) and 95% confidence interval (95% CI). The significance of differences in variable influencing OS was estimated by the log-rank test (Mantel–Cox), while Gray's test[50] was used to assess, in univariate analyses, differences between cumulative incidences. A *p*-value lower than 0.05 was considered to be statistically significant.

Disclosure of potential conflict of interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by grants from: AIRC (IG-17047 to I.A; Special Grant "5xmille"-9962 to F. Locatelli, L.M; "My first AIRC" grant 15925 to A.B.), Ministero della Salute (RF-2010-2308270 to I.A; RF-2010-2316606 to F. Locatelli; Ricerca Corrente to F. Locatelli), Regione Lazio (Grant FILAS to F. Locatelli), and Ministero dell'Istruzione, Università e Ricerca (Grant Progetto di Rilevante Interesse Nazionale, PRIN 2010, to F. Locatelli). A.Z. is an F.I.R.C. fellow.

Data and materials availability

Pride database, Project Name: Human $\gamma\delta$ T-cell, LC-MS/MS, Project accession: PXD002629.

ORCID

B. De Angelis http://orcid.org/0000-0002-7938-737X

References

1. Henslee-Downey PJ, Abhyankar SH, Parrish RS, Pati AR, Godder KT, Neglia WJ, Goon-Johnson KS, Geier SS, Lee CG, Gee AP. Use of partially mismatched related donors extends access to allogeneic marrow transplant. Blood 1997; 89:3864-72; PMID:9160695
2. Aversa F, Terenzi A, Tabilio A, Falzetti F, Carotti A, Ballanti S, Felicini R, Falcinelli F, Velardi A, Ruggeri L et al. Full haplotype-mismatched hematopoietic stem-cell transplantation: a phase II study in patients with acute leukemia at high risk of relapse. J Clin Oncol 2005; 23:3447-54; PMID:15753458; http://dx.doi.org/10.1200/JCO.2005.09.117
3. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, Posati S, Rogaia D, Frassoni F, Aversa F et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science 2002; 295:2097-100; PMID:11896281; http://dx.doi.org/10.1126/science.1068440
4. Locatelli F, Merli P, Rutella S. At the Bedside: Innate immunity as an immunotherapy tool for hematological malignancies. J Leukocyte Biol 2013; 94:1141-57; PMID:24096380; http://dx.doi.org/10.1189/jlb.0613343
5. Bertaina A, Merli P, Rutella S, Pagliara D, Bernardo ME, Masetti R, Pende D, Falco M, Handgretinger R, Moretta F et al. HLA-haploidentical stem cell transplantation after removal of alpha-beta+ T and B cells in children with nonmalignant disorders. Blood 2014; 124:822-6; PMID:24869942; http://dx.doi.org/10.1182/blood-2014-03-563817
6. Schumm M, Lang P, Bethge W, Faul C, Feuchtinger T, Pfeiffer M, Vogel W, Huppert V, Handgretinger R. Depletion of T-cell receptor α/β and CD19 positive cells from apheresis products with the CliniMACS device. Cytotherapy 2013; 15:1253-8; PMID:23993299; http://dx.doi.org/10.1016/j.jcyt.2013.05.014
7. Bonneville M, O'Brien RL, Born WK. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. Nat Rev Immunol 2010; 10:467-78; PMID:20539306; http://dx.doi.org/10.1038/nri2781
8. Elmaagacli AH, Steckel NK, Koldehoff M, Hegerfeldt Y, Trenschel R, Ditschkowski M, Christoph S, Gromke T, Kordelas L, Ottinger HD et al. Early human cytomegalovirus replication after transplantation is associated with a decreased relapse risk: evidence for a putative virus-versus-leukemia effect in acute myeloid leukemia patients. Blood 2011; 118:1402-12; PMID:21540462; http://dx.doi.org/10.1182/blood-2010-08-304121
9. Knight A, Madrigal AJ, Grace S, Sivakumaran J, Kottaridis P, Mackinnon S, Travers PJ, Lowdell MW. The role of Vdelta2-negative gammadelta T cells during cytomegalovirus reactivation in recipients of allogeneic stem cell transplantation. Blood 2010; 116:2164-72; PMID:20576814; http://dx.doi.org/10.1182/blood-2010-01-255166
10. Scheper W, van Dorp S, Kersting S, Pietersma F, Lindemans C, Hol S, Heijhuurs S, Sebestyen Z, Gründer C et al. Gammadelta T cells elicited by CMV reactivation after allo-SCT cross-recognize CMV and leukemia. Leukemia 2013; 27:1328-38; PMID:23277330; http://dx.doi.org/10.1038/leu.2012.374
11. Vantourout P, Hayday A. Six-of-the-best: unique contributions of gammadelta T cells to immunology. Nat Rev Immunol 2013; 13:88-100; PMID:23348415; http://dx.doi.org/10.1038/nri3384
12. Brandes M, Willimann K, Bioley G, Levy N, Eberl M, Luo M, Tampé R, Lévy F, Romero P, Moser B. Cross-presenting human gammadelta T cells induce robust CD8+ alphabeta T cell responses. Proc Natl Acad Sci U S A 2009; 106:2307-12; PMID:19171897; http://dx.doi.org/10.1073/pnas.0810059106
13. Kabelitz D, Kalyan S, Oberg HH, Wesch D. Human Vdelta2 versus non-Vdelta2 gammadelta T cells in antitumor immunity. Oncoimmunology 2013; 2:e23304; PMID:23802074; http://dx.doi.org/10.4161/onci.23304
14. Dechanet J, Merville P, Lim A, Retiere C, Pitard V, Lafarge X, Michelson S, Méric C, Hallet MM, Kourilsky P et al. Implication of gammadelta T cells in the human immune response to cytomegalovirus. J Clin Invest 1999; 103:1437-49; PMID:10330426; http://dx.doi.org/10.1172/JCI5409
15. Airoldi I, Bertaina A, Prigione I, Zorzoli A, Pagliara D, Cocco C, Meazza R, Loiacono F, Lucarelli B, Bernardo ME et al. Gammadelta T-cell reconstitution after HLA-haploidentical hematopoietic transplantation depleted of TCR-alphabeta+/CD19+ lymphocytes. Blood 2015; 125:2349-58; PMID:25612623; http://dx.doi.org/10.1182/blood-2014-09-599423
16. Couzi L, Lafarge X, Pitard V, Neau-Cransac M, Dromer C, Billes MA, Lacaille F, Moreau JF, Merville P, Déchanet-Merville J.

Gamma-delta T cell expansion is closely associated with cytomegalovirus infection in all solid organ transplant recipients. Transplant Int 2011; 24:e40-2; PMID:21463369; http://dx.doi.org/10.1111/j.1432-2277.2010.01181.x
17. Dieli F, Gebbia N, Poccia F, Caccamo N, Montesano C, Fulfaro F, Arcara C, Valerio MR, Meraviglia S, Di Sano C et al. Induction of gammadelta T-lymphocyte effector functions by bisphosphonate zoledronic acid in cancer patients in vivo. Blood 2003; 102:2310-1; PMID:12959943; http://dx.doi.org/10.1182/blood-2003-05-1655
18. Gertner-Dardenne J, Fauriat C, Vey N, Olive D. Immunotherapy of acute myeloid leukemia based on gammadelta T cells. Oncoimmunol 2012; 1:1614-6; PMID:23264912; http://dx.doi.org/10.4161/onci.21512
19. Wilhelm M, Kunzmann V, Eckstein S, Reimer P, Weissinger F, Ruediger T, Tony HP. Gammadelta T cells for immune therapy of patients with lymphoid malignancies. Blood 2003; 102:200-6; PMID:12623838; http://dx.doi.org/10.1182/blood-2002-12-3665
20. Stachnik A, Yuen T, Iqbal J, Sgobba M, Gupta Y, Lu P, Colaianni G, Ji Y, Zhu LL, Kim SM et al. Repurposing of bisphosphonates for the prevention and therapy of nonsmall cell lung and breast cancer. Proc Natl Acad Sci U S A 2014; 111:17995-8000; PMID:25453078; http://dx.doi.org/10.1073/pnas.1421422111
21. Godder KT, Henslee-Downey PJ, Mehta J, Park BS, Chiang KY, Abhyankar S, Lamb LS. Long term disease-free survival in acute leukemia patients recovering with increased gammadelta T cells after partially mismatched related donor bone marrow transplantation. Bone Marrow Transplant 2007; 39:751-7; PMID:17450185; http://dx.doi.org/10.1038/sj.bmt.1705650
22. Lamb LS, Jr, Henslee-Downey PJ, Parrish RS, Godder K, Thompson J, Lee C, Gee AP. Increased frequency of TCR gamma delta + T cells in disease-free survivors following T cell-depleted, partially mismatched, related donor bone marrow transplantation for leukemia. J Hematother 1996; 5:503-9; PMID:8938522; http://dx.doi.org/10.1089/scd.1.1996.5.503
23. Meraviglia S, Eberl M, Vermijlen D, Todaro M, Buccheri S, Cicero G, D'Asaro M, Orlando V, Scarpa F, Roberts A et al. In vivo manipulation of Vgamma9Vdelta2 T cells with zoledronate and low-dose interleukin-2 for immunotherapy of advanced breast cancer patients. Clin Exp Immunol 2010; 161:290-7; PMID:20491785; http://dx.doi.org/10.1111/j.1365-2249.2010.04167
24. Caccamo N, Meraviglia S, Ferlazzo V, Angelini D, Borsellino G, Poccia F, Salerno A. Differential requirements for antigen or homeostatic cytokines for proliferation and differentiation of human Vgamma9Vdelta2 naive, memory and effector T cell subsets. Eur J Immunol 2005; 35:1764-72; PMID:15915537; http://dx.doi.org/10.1002/eji.200525983
25. Shen Y, Zhou D, Qiu L, Lai X, Simon M, Shen L, Kou Z, Wang Q, Jiang L, Estep J et al. Adaptive immune response of Vgamma2Vdelta2+ T cells during mycobacterial infections. Science 2002; 295:2255-8; PMID:11910108; http://dx.doi.org/10.1126/science.1068819
26. Berenson JR, Hillner BE, Kyle RA, Anderson K, Lipton A, Yee GC, Biermann JS. American Society of Clinical Oncology clinical practice guidelines: the role of bisphosphonates in multiple myeloma. J Clin Oncol 2002; 20:3719-36; PMID:12202673; http://dx.doi.org/10.1200/JCO.2002.06.037
27. Gnant M. Can oral bisphosphonates really reduce the risk of breast cancer in healthy women? J Clin Oncol 2010; 28:3548-51; PMID:20567005; http://dx.doi.org/10.1200/JCO.2010.29.6327
28. Michaelson MD, Smith MR. Bisphosphonates for treatment and prevention of bone metastases. J Clin Oncol 2005; 23:8219-24; PMID:16278476; http://dx.doi.org/10.1200/JCO.2005.02.9579
29. Russell HV, Groshen SG, Ara T, DeClerck YA, Hawkins R, Jackson HA, Daldrup-Link HE, Marachelian A, Skerjanec A, Park JR et al. A phase I study of zoledronic acid and low-dose cyclophosphamide in recurrent/refractory neuroblastoma: a new approaches to neuroblastoma therapy (NANT) study. Pediatric Blood Cancer 2011; 57:275-82; PMID:21671363; http://dx.doi.org/10.1002/pbc.22821
30. Kulak NA, Pichler G, Paron I, Nagaraj N, Mann M. Minimal, encapsulated proteomic-sample processing applied to copy-number estimation in eukaryotic cells. Nat Methods 2014; 11:319-24; PMID:24487582; http://dx.doi.org/10.1038/nmeth.2834
31. Hahne H, Pachl F, Ruprecht B, Maier SK, Klaeger S, Helm D, Médard G, Wilm M, Lemeer S, Kuster B. DMSO enhances electrospray response, boosting sensitivity of proteomic experiments. Nat Methods 2013; 10:989-91; http://dx.doi.org/10.1038/nmeth.2610
32. Pirmoradian M, Budamgunta H, Chingin K, Zhang B, Astorga-Wells J, Zubarev RA. Rapid and deep human proteome analysis by single-dimension shotgun proteomics. Mol Cell Proteomics 2013; 12:3330-8; http://dx.doi.org/10.1074/mcp.O113.028787
33. Mann M, Kulak NA, Nagaraj N, Cox J. The coming age of complete, accurate, and ubiquitous proteomes. Mol Cell 2013; 49:583-90; PMID:23438854; http://dx.doi.org/10.1016/j.molcel.2013.01.029
34. Neuhauser N, Nagaraj N, McHardy P, Zanivan S, Scheltema R, Cox J, Mann M. High performance computational analysis of large-scale proteome data sets to assess incremental contribution to coverage of the human genome. J Proteome Res 2013; 12:2858-68; PMID:23611042; http://dx.doi.org/10.1021/pr400181q
35. Hornburg D, Drepper C, Butter F, Meissner F, Sendtner M, Mann M. Deep proteomic evaluation of primary and cell line motoneuron disease models delineates major differences in neuronal characteristics. Mol Cell Proteomics 2014; 13:3410-20; http://dx.doi.org/10.1074/mcp.M113.037291
36. Tyanova S, Albrechtsen R, Kronqvist P, Cox J, Mann M, Geiger T. Proteomic maps of breast cancer subtypes. Nat Commun 2016; 7:10259; PMID:26725330; http://dx.doi.org/10.1038/ncomms10259
37. Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, Fridman WH, Pagès F, Trajanoski Z, Galon J. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics 2009; 25:1091-3; PMID:19237447; http://dx.doi.org/10.1093/bioinformatics/btp101
38. Dieli F, Poccia F, Lipp M, Sireci G, Caccamo N, Di Sano C, Salerno A. Differentiation of effector/memory Vdelta2 T cells and migratory routes in lymph nodes or inflammatory sites. J Exp Med 2003; 198:391-7; PMID:12900516; http://dx.doi.org/10.1084/jem.20030235
39. Angelini DF, Borsellino G, Poupot M, Diamantini A, Poupot R, Bernardi G, Poccia F, Fournié JJ, Battistini L. FcgammaRIII discriminates between 2 subsets of Vgamma9Vdelta2 effector cells with different responses and activation pathways. Blood 2004; 104:1801-7; PMID:15178578; http://dx.doi.org/10.1182/blood-2004-01-0331
40. Li D, Song L, Fan Y, Li X, Li Y, Chen J, Zhu F, Guo C, Shi Y, Zhang L. Downregulation of TIPE2 mRNA expression in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. Clin Immunol 2009; 133:422-7; PMID:19748832; http://dx.doi.org/10.1016/j.clim.2009.08.014
41. Zhang S, Zhang Y, Wei X, Zhen J, Wang Z, Li M, Miao W, Ding H, Du P, Zhang W et al. Expression and regulation of a novel identified TNFAIP8 family is associated with diabetic nephropathy. Biochim Et Biophys Acta 2010; 1802:1078-86; PMID:20699119; http://dx.doi.org/10.1016/j.bbadis.2010.08.003
42. Xi W, Hu Y, Liu Y, Zhang J, Wang L, Lou Y, Qu Z, Cui J, Zhang G, Liang X et al. Roles of TIPE2 in hepatitis B virus-induced hepatic inflammation in humans and mice. Mol Immunol 2011; 48:1203-8; PMID:21466895; http://dx.doi.org/10.1016/j.molimm.2011.03.002
43. Sun H, Lou Y, Porturas T, Morrissey S, Luo G, Qi J, Ruan Q, Shi S, Chen YH. Exacerbated experimental colitis in TNFAIP8-Deficient mice. J Immunol 2015; 194:5736-42; http://dx.doi.org/10.4049/jimmunol.1401986
44. Lou Y, Liu S. The TIPE (TNFAIP8) family in inflammation, immunity, and cancer. Mol Immunol 2011; 49:4-7; PMID:21924498; http://dx.doi.org/10.1016/j.molimm.2011.08.006
45. Babbe H, Chester N, Leder P, Reizis B. The Bloom's syndrome helicase is critical for development and function of the alphabeta T-cell lineage. Mol Cell Biol 2007; 27:1947-59; PMID:17210642; http://dx.doi.org/10.1128/MCB.01402-06
46. Ziegler H, Welker C, Sterk M, Haarer J, Rammensee HG, Handgretinger R, Schilbach K. Human peripheral CD4(+) Vdelta1(+) gammadeltaT Cells can develop into alphabetaT cells. Frontiers

Immunol 2014; 5:645; PMID:25709606; http://dx.doi.org/10.3389/fimmu.2014.00645

47. Kalpan EL, Meier P. Non parametric estimation from incomplete observations. J Am Stat Assoc 1958; 53:467-81.
48. Gooley TA, Leisenring W, Crowley J, Storer BE. Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. Statistics Med 1999; 18:695-706; PMID:10204198; http://dx.doi.org/10.1002/(SICI)1097-0258(19990330)18:6%3c695::AID-SIM60%3e3.0.CO;2-O
49. Pepe MS, Longton G, Pettinger M, Mori M, Fisher LD, Storb R. Summarizing data on survival, relapse, and chronic graft-versus-host disease after bone marrow transplantation: motivation for and description of new methods. Br J Haematol 1993; 83:602-7; PMID:8518176; http://dx.doi.org/10.1111/j.1365-2141.1993.tb04697.x
50. Gray RJ. A class of K-sample tests for comparing the cumulative incidence of a competing risk. Ann Statist 2006; 16:1141-54; http://dx.doi.org/10.1214/aos/1176350951