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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

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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 V$\alpha$2-cell differentiation, paralleled by increased cytotoxicity of both V$\delta$1 and V$\delta$2 cells against primary leukemia blasts was associated with ZOL treatment. Cytotoxic activity was further increased in V$\delta$2 cells, but not in V$\delta$1 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

Introduction

Hematopoietic stem cell transplantation (HSCT) from an HLA-haploidentical relative (haplo-HSCT) offers an immediate transplantation treatment virtually to any patient in need of an allograft and lacking a suitable matched donor. The use of haplo-HSCT was initially hampered by the risk of graft rejection, graft-versus-host disease (GvHD), and delayed immune recovery leading to an increased incidence of opportunistic infections. Human $\gamma\delta$ T cells orchestrate cellular activities of both innate and adaptive immunity, and, unlike $\alpha\beta^+$ T lymphocytes, recognize tumors in a MHC-independent manner and do not cause GvHD. 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. Among circulating $\gamma\delta$ T cells, there is a major subset expressing V$\delta$2 chain and a minor subset expressing V$\delta$1 chain. Both subsets share antitumor properties, but V$\delta$1 cells reside also within epithelial tissues, especially at sites of CMV replication, and may undergo selective expansion in transplanted patients upon...
cytomegalovirus (CMV) reactivation.\textsuperscript{8-10,15,16} The V\textdelta2 population recognizes non-peptide phospho-antigens, may be expanded and activated \textit{ex vivo} and \textit{in vivo} by aminobisphosphonates, such as zoledronic acid (ZOL),\textsuperscript{17} thus resulting in an attractive immuno-therapeutic tool against cancer.

Current adoptive immunotherapy approaches are limited to the V\textdelta2 cell subpopulation due to limited expansion of V\textdelta1 cells to reach numbers sufficient for clinical applications. ZOL infusion resulted in objective clinical responses against both solid and hematologic tumors,\textsuperscript{17-20} but was not curative as infusion resulted in objective clinical responses against both solid and hematologic tumors,\textsuperscript{17-20} but was not curative as

43 children receiving a B- and \alpha\beta T-cell-depleted haplo-HSCT after ZOL infusion.

Results

\textit{\gamma\delta 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 \gamma\delta T-cell lineage (mean 61\% of gated CD3\(^+\) lymphocytes, range from 34 to 91\%). Afterwards, the \alpha\beta T-cell population gradually increased (not shown) and the \gamma\delta T-cell population decreased over time (Fig. 1A), as already reported for a different cohort of leukemia patients that we previously published,\textsuperscript{15} and who had received the same type of graft without being treated with ZOL (controls). Comparative analyses of \gamma\delta T cells, V\delta1, and V\delta2 subsets in controls and in ZOL-treated pts, revealed that, 3 mo after HSCT, a significant increase of the percentage of V\delta1 cells (Fig. 1B, left panel), paralleled by a decrease of the percentage of V\delta2 cell subset occurred (Fig. 1B, right panel). Such behavior was observed until month 6, when the percentage of \gamma\delta 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 \gamma\delta T cells and of their subsets.

We recently described\textsuperscript{15} that \gamma\delta T cells from recipients of haplo-HSCT express cytotoxic molecules, as well as produce IFN\gamma. Thus, we tested whether ZOL infusion maintained \gamma\delta T cells fully functional to lyse target cells. As shown in Fig. 1C, \gamma\delta T cells from recipients of haplo-HSCT treated with ZOL expressed perforin, granzyme-B and, upon calcium ionophore/PMA stimulation, produced IFN\gamma. Next, we asked whether clinical outcome may be influenced by the percentage of peripheral V\gamma9V\delta2 T cells, as observed by others\textsuperscript{23} in patients with advanced breast cancer treated with ZOL. To this end, we compared the percentage of peripheral V\gamma9V\delta2 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 V\gamma9V\delta2 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 V\delta1 and V\delta2 subsets in circulating \gamma\delta T cells

Next, we evaluated whether the increase in the percentage of V\delta1 cells and the decrease of V\delta2 cells observed over time was dependent exclusively on CMV reactivation, as already reported,\textsuperscript{10,15} and/or also on ZOL infusion. Thus, we analyzed the percentage of V\delta1 and V\delta2 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 V\delta1 cells significantly increased and V\delta2 cells decreased upon the second/third, but not the first ZOL treatment. In particular, the V\delta1 subset became the main circulating \gamma\delta 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 V\delta1 population was dependent on ZOL exposure and not mainly on virus reactivation (Fig. 2B, white plots). However, in patients experiencing CMV reactivation, the V\delta1 population was expanded and represented the main \gamma\delta T-cell subset in both ZOL-treated and untreated patients (Fig. 2B, gray plots and 2C).

ZOL infusion induces differentiation and increases cytotoxicity of V\delta2 cells in vivo

Phenotypic analyses revealed that, before ZOL treatment, 14.5\% of V\delta2 lymphocytes were \textit{naive}, 44.9\% were CM, 22.6\% EM, and 15.1\% TD (Fig. 3A). The percentage of CM V\delta2 cells significantly decreased and that of TD increased starting from the first ZOL administration (Fig. 3A). The \textit{naive} and EM V\delta2 populations were not affected by ZOL treatment.

Next, we investigated whether V\delta2 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, V\delta2 cells obtained from patients before ZOL treatment and cultured with AML/ALL blasts, expressed CD107a on the cell surface (mean percentage of CD107a\(^+\) cells in gated V\delta2 subset being 11.56). \textit{In vitro} pre-treatment of blasts with ZOL significantly increased the proportion of CD107a\(^+\) cells in gated V\delta2 (mean percentage
being 22.57) cells, this suggesting that such γδ T-cell population exerts cytotoxic functions when the target expresses high levels of phosphoantigens. More importantly, γδ 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
target cells had been previously treated with ZOL. By contrast, V\(d_2\) cells from patients treated three times or more with ZOL significantly increased their cytotoxic activity against untreated leukemia cells, especially when compared to V\(d_2\) cells obtained from patients treated only once (Fig. 3). No significant differences were observed in CD107a surface expression in V\(d_2\) cells from patients treated 3/5 times and challenged with AML or ALL cells treated with ZOL versus V\(d_2\) cells cultured with untreated AML or ALL blasts (Figs. 3B and C). Thus, V\(d_2\) 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 V\(d_1\) 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 V\(d_1\) 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 V\(d_1\) 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 V\(d_1\) 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 V22 and V61 cells in ZOL-treated patients. (A) Percentage of naïve, CM, EM, and TD subsets was evaluated, by flow cytometry, in gated CD3⁺γδ⁺V22⁺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 γδ⁺V22⁺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⁺γδ⁺V61⁺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 γδ⁺V61⁺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 FcγR1γ 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 γδ 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 γδ T cells before and after the first ZOL treatment. The log10 fold change for each protein is plotted against the log10 of the p-value. The polygon (FDR < 0.05, S0 > 0.5) splits the significant proteins from those that are not modulated. The γδ 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 γδ 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 γδ 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 γδ 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 γδ 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 γδ T cells from pt #27 before treatment versus γδ 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 γδ 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 γδ 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 γδ 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**

\( \gamma d \) 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. \( \gamma d \) T cells: (i) recognize tumor antigens in MHC-independent manner; (ii) have endogenous cytotoxicity; (iii) produce cytokines useful to mount antitumor and antiviral responses; (iv) may be \textit{ex vivo} expanded and activated with ZOL; and (v) may develop immunological memory. These features render \( \gamma d \) 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 the Food and Drug Administration for the treatment of metastatic bone involvement by hematopoietic tumors, such as multiple myeloma, and by solid tumors, including breast and prostate cancers. In children, a phase I study of ZOL in recurrent/refractory neuroblastoma showed clinical and biological response, with mild toxicity.

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 \( \gamma d \) T cells and patients’ outcomes. Here, we report the effects exerted \textit{in vivo} by ZOL on \( \gamma d \) T cells, using classical phenotypical and functional assays, synergistically integrated with innovative proteomic tools of sample preparation, analytical conditions, high-resolution mass spectrometry, statistical and network analysis. 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, sensitivity, and specificity. The \textit{in vivo} evolution of \( \gamma d \) 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 \( \text{V} \delta \text{1} \) and \( \text{V} \delta \text{2} \) subsets. ZOL influenced, unexpectedly, the phenotype and function not only of \( \text{V} \delta \text{2} \) cells, which selectively recognize phosphoantigens, but also of the \( \text{V} \delta \text{1} \) population. In particular, the first treatment with ZOL induced the differentiation of \( \text{V} \delta \text{2} \) cells, which showed a maturation trend, moving from a CM to an EM/TD phenotype. This maturation was associated with immediate effector functions, a result supported by our functional experiments highlighting a boost of \( \text{V} \delta \text{2} \) cell cytotoxicity against primary leukemia cell blasts, irrespectively of their phosphoantigen expression. The anti-proliferative effect of ZOL on total \( \gamma d \) T cells, identified by proteomic studies, was reflected by the decrease of the \( \text{V} \delta \text{2} \) population starting from month 3 after HSCT. By contrast, the percentage of \( \text{V} \delta \text{1} \) subset increased over time upon ZOL infusion, irrespectively of CMV reactivation. The percentage of \( \text{V} \delta \text{2} \) 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 \( V \gamma 9V \delta 2 \) cells induced by aminobisphosphonates in patients with malignancies were associated with good prognosis.

Phenotypic and functional studies delineated a route of activation in \( \gamma d \) 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 \( \gamma d \) T cells upon each individual ZOL infusion. To date, most of them have not been associated with \( \gamma d \) T-cell functions and deserve future, specific investigations. Nonetheless, induction of TNFAIP8L2/TIE2 and BLM captured our attention, since their presence may be relevant in the setting of HSCT. TIE2 is a cytoplasmic protein predominantly expressed in immune cells and especially in T lymphocytes; abnormal expression of TIE2 is implicated in systemic autoimmunity, diabetic nephropathy, and hepatitis B. In addition, TIE2-knockout mice developed a severe colitis, with enhanced leukocyte infiltration, bacterial invasion, and inflammatory cytokine in the colon.

A key protein that signed the prototype of \( \gamma d \) T cells in transplanted patients treated twice with ZOL is BLM, important in development, maintenance, and function of \( \alpha \beta \) T lymphocytes. 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 \( \text{Blm} \)-deficient mice. It was recently reported that a minor subset of peripheral \( \text{V} \delta \text{1} \) cells that express CD4+ in association with stemness/progenitor markers may transdifferentiate into \( \alpha \beta \) T cells. This demonstration, in addition with our finding that ZOL increased the \( \text{V} \delta \text{1} \) percentage and induced BLM in \( \gamma d \) T cells, lets envisage a peculiar scenario in which ZOL may induce \textit{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, effectively protecting patients from GvHD occurrence and improving OS. ZOL treatment caused multifunctional beneficial effects on \( \gamma d \) T cells, our results suggesting new proteomic keys to test the responsiveness of patients to this treatment.
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 TCRfi-gd T-cell phenotype, functionally studied at day 7, revealed that the majority of lymphocytes in PB were represented by BCP-ALL, CR2 and in 12 pts (28%), these being time points at which all children had already obtained engraftment of donor hematopoiesis and had already received an allograft from an unrelated donor.

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 after relapse                       | 6            |
| Pt #4 (M)         | 11          | BCP-ALL, CR3                              | 182                                         | +               | alive                                    | 4            |
| Pt #5 (F)         | 13          | BCP-ALL, CR1                              | 195                                         | +               | alive                                    | 3            |
| 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                                         | +               | alive                                    | 3            |
| Pt #10 (M)        | 6           | BCP-ALL, CR2                              | 225                                         | –               | alive                                    | 3            |
| Pt #11 (M)        | 15          | AML, CR1                                 | 457                                         | –               | alive                                    | 4            |
| 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.

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 γδ 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. γδ 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 αβ (clone B3);
Table 2. Graft composition.

| Patients | CD34+Kg (10^6) | TCR-αβKg (10^6) | TCR-γδKg (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           | 9.67            | 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            | 0.026          |
| 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 γδ; 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 Bioscences, 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 γδ T cells

PBMC were enriched by Ficoll-Hypaque (Sigma Aldrich) density gradient centrifugation. We acquired at least 10^6 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 γδ 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 γδ T-cell subsets were evaluated in gated CD3+/Vδ1+ or CD3+/Vδ2+ lymphocytes.

Degranulation assay

Degranulation assay was performed by co-culturing 10⁵ effectors (E) and 10⁵ 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 γδ, -Vδ1, -Vδ2, and CD107a analyzed in gated Vδ1 or Vδ2 γδ T cells, by flow cytometry. At least 1.5 × 10⁶ events were acquired.

Evaluation of the protein expression profile in circulating γδ T cells before and after treatment with ZOL

γδ 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 × 10⁵ γδ 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 × 10⁵ γδ T cells were
purified. Cells were lysed and the extracted proteins subjected to proteolysis employing an in-StageTip method.40 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).58,49 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 test10 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.

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Data and materials availability

Pride database, Project Name: Human γδ T-cell, LC-MS/MS, Project accession: PXD002629.

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References

1. Hensley-Downey JP, Abhyankar SH, Parrish RS, Pati AR, Godder KT, Neglia WJ, Goon-Johnson KS, Geier SS, Lee GC, Ge A.P. Use of partially mismatched related donors extends access to allogeneic marrow transplantation. Blood 1997; 89:3864-72; PMID:9160965

2. Aversa F, Terezni A, Tabillo 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, Capani 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, Trenscher 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 leukaemia 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, Sivakumar J, Kottaridis P, Mackinnon S, Travers P, Lowdell MW. The role of Vdelta2-negative gammadelta T cells during cytomegalovirus replication 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. Schepers 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 leukaemia. 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 immunity. 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, Levy F, Romero P, Moser B. Cross-presenting human gammadelta T cells induce robust CD8+ alpha-beta 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, Wessch 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.e23304

14. Dechanet J, Merville P, Lim A, Retiere C, Pitard V, Lafarge X, Michelon S, Méric C, Hallet MM, Kourilsky P et al. Implication of gamma-delta T and B cells in children with nonmalignant disorders. 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, Mezza 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, Décéanet-Merville J.
Gamma-delta T cell expansion is closely associated with cytomegalovirus infection in all solid organ transplant recipients. Transplant Int 2011; 24:e02-0; 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, Fularo F, Arcara C, Valerio MR, Meraviglia S, Di Sano C et al. Induction 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.1421241111

18. Gartner-Dardenne J, Fauriat C, Vey N, Olive D. Immunotherapy of acute myeloid leukemia based on gammadelta T cells. Oncotarget 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; 102:2310-6; 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 bone metastases. J Clin Oncol 2010; 28:3548-51; PMID:20567005; http://dx.doi.org/10.1200/JCO.2010.29.6327

21. Godder KT, Henslee-Downey PJ, Mehta J, Park BS, Chiang KY, Godder KT, Henslee-Downey PJ, Mehta J, Park BS, Chiang KY. 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

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. Bone Marrow Transplant 2007; 39:751-7; PMID:17450185; http://dx.doi.org/10.1038/sj.bmj.1705650

23. Meraviglia S, Eberl M, Vermilen 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 zolendronic acid in vivo. Exp Immunol 2010; 161:290-7; PMID:20491785; http://dx.doi.org/10.1111/j.1432-2277.2010.01181.x

24. Caccamo N, Meraviglia S, Ferlazzo V, Angelini D, Borsellino G, Arcara C, Valerio MR, Meraviglia S, Di Sano C et al. Induction 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.1421241111

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. Grant M. Can oral bisphosphonates really reduce the risk of breast cancer in healthy women? J Clin Oncol 2010; 28:5348-51; 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, Groschen SG, Atra T, DeClerck VA, Hawkins R, Jackson HA, Daldup-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 approach 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, Medard 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, Badamganta 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. Neuhauer 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, Drexper 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
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