Identification of a panel of ten cell surface protein antigens associated with immunotargeting of leukemias and lymphomas by peripheral blood γδ T cells

Anita Q. Gomes,[1,2,3] Daniel V. Correia,[1,2] Ana R. Grosso,[1] Telma Lança,[1] Cristina Ferreira,[4] João F. Lacerda,[4] João T. Barata,[1] Maria Gomes da Silva,[5] and Bruno Silva-Santos[1,2]

[1]Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa; [2]Instituto Gulbenkian de Ciência, Oeiras; [3]Escola Superior de Tecnologia da Saúde de Lisboa, and [4]Hospital de Santa Maria-CHLN, Lisboa, and [5]Instituto Português de Oncologia de Lisboa, Francisco Gentil, Portugal

AQG and DVC contributed equally to this manuscript.

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Correspondence: Bruno Silva-Santos, Unidade de Imunologia Molecular, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Avenida Professor Egas Moniz, 1649-028 Lisboa, Portugal. E-mail: bssantos@fm.ul.pt

The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

Vγ9Vδ2 T lymphocytes are regarded as promising mediators of cancer immunotherapy due to their capacity to eliminate multiple experimental tumors, particularly within those of hematopoietic origin. However, Vγ9Vδ2 T-cell based lymphoma clinical trials have suffered from the lack of biomarkers that can be used as prognostic of therapeutic success.

Design and Methods

We have conducted a comprehensive study of gene expression in acute lymphoblastic leukemias and non-Hodgkin's lymphomas, aimed at identifying markers of susceptibility versus resistance to Vγ9Vδ2 T cell-mediated cytotoxicity. We employed cDNA microarrays and quantitative real-time PCR to screen 20 leukemia and lymphoma cell lines, and 23 primary hematopoietic tumor samples. These data were analyzed using state-of-the-art bioinformatics, and gene expression patterns were correlated with susceptibility to Vγ9Vδ2 T cell mediated cytolysis *in vitro*.

Results

We identified a panel of 10 genes encoding cell surface proteins that were statistically differentially expressed between "γδ-susceptible" and "γδ-resistant" hematopoietic tumors. Within this panel, 3 genes (ULBP1, TFR2 and IFITM1) were associated with increased susceptibility to Vγ9Vδ2 T-cell cytotoxicity, whereas the other 7 (CLEC2D, NRP2, SELL, PKD2, KCNK12, ITGA6 and SLAMF1) were enriched in resistant tumors. Furthermore, some of these candidates displayed a striking variance of expression among primary follicular lymphomas and T-cell acute lymphoblastic leukemias.

Conclusions

Our results suggest that hematopoietic tumors display a highly variable repertoire of surface proteins that can impact on Vγ9Vδ2 cell-mediated immunotargeting. The prognostic value of the proposed markers can now be evaluated in upcoming Vγ9Vδ2 T cell-based lymphoma/leukemia clinical trials.

Key words: biomarkers, Vγ9Vδ2 T-lymphocytes, hematopoietic tumors, lymphoma cell lines.

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Introduction

γδ T lymphocytes display potent innate anti-tumor activity in both humans[1] and mice.[2,3] For example, mice genetically devoid of γδ T cells displayed increased susceptibility to skin tumor development induced experimentally by carcinogens,[2,3] and to transgenic adenocarcinoma of the mouse prostate model (TRAMP).[4] More importantly, murine γδ T cells were shown to prevent (through perforin-mediated cytotoxicity) the development of spontaneous B-cell lymphomas.[5]

The major γδ T-cell subset in human peripheral blood, Vγ9Vδ2 T lymphocytes, exert potent cytotoxicity towards tumor cell lines upon activation with small non-peptidic prenyl pyrophosphate intermediates of isoprenoid biosynthesis.[6] We and others have shown that, among such "phosphoantigens", 4-hydroxy-3-methyl-but-2-enylpyrophosphate (HMB-PP), a metabolite found in *Eubacteria* and *Protozoa*, is a very potent agonist of the Vγ9Vδ2 T-cell receptor (TCR) that promotes cytotoxicity and the secretion of anti-tumor cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor α (TNF-α).[6,7]

Phosphoantigen-activated Vγ9Vδ2 T cells can kill various solid tumor cell lines,[1] and a particularly large number of hematopoietic cell-derived tumors,[7-9] as well as freshly isolated tumor cells from patients with follicular B-cell lymphoma or chronic lymphocytic leukemia (CLL).[10]

The well-established anti-tumor activity of Vγ9Vδ2 T cells has been recently explored in clinical trials for solid/epithelial[11-13] or liquid/hematopoietic tumors (14-16), which were collectively promising even though they showed limited success. The lack of response to therapy of some patients was attributed to deficient expansion of effector Vγ9Vδ2 T cells.[11,13,14] However, a large proportion of patients exhibiting significant and sustained *in vivo* activation and proliferation of Vγ9Vδ2 T cells also failed to respond to treatment. Thus, in both prostate carcinoma[11] and non-Hodgkin's lymphoma,[14] objective responses (partial remissions) were observed in just 33% of the patients who activated/expanded their Vγ9Vδ2 T cells. These data emphasize the need for tumor biomarkers with prognostic value for γδ peripheral blood lymphocyte (γδ-PBL)-mediated immunotherapy.

Here we have conducted a comprehensive genome-wide expression study aimed at identifying lymphoma/leukemia markers of susceptibility or resistance to γδ-PBL cytotoxicity. We set up an experimental system consisting of lymphoma/leukemia cell lines with various degrees of susceptibility to γδ-PBL-mediated lysis, and performed comparative cDNA microarray analyses to characterize their gene expression profiles. These were validated through bioinformatics and quantitative real-time PCR (RT-qPCR), allowing us to define a panel of 10 candidate biomarkers whose expression displayed very marked variability among non-Hodgkin's lymphoma and acute lymphoblastic leukemia patients.

Design and Methods

In vitro *cultures of human γδ-PBL and tumor cell lines*

Peripheral blood was collected from healthy volunteers and peripheral blood mononuclear cells (PBMCs) were isolated as previously described.[7] γδ–PBL were expanded from isolated PBMCs for 12 days in RPMI 1640 complete media[7] supplemented with 100 U/mL of rhIL-2 (Roche Applied Science) and 1 nM HMB-PP (4-hydroxy-3-methyl-but-2-enylpyrophosphate) (Sup-RPMI). The percentage of $V\gamma9^+$ T cells in peripheral blood increased from 3-14% at day 0 to 90-98% at day 12 (*Online Supplementary Figure S1*). All tumor cell lines were cultured in complete 10% RPMI-1640 as previously described.[7]

Leukemia and lymphoma primary samples

Pediatric B- or T-cell acute lymphoblastic leukemia cells containing high (> 80%) leukemia involvement were obtained from the peripheral blood and/or the bone marrow of patients at presentation after informed consent and institutional review board approval (Instituto Português de Oncologia, Lisbon, Portugal) had been obtained. Fresh leukemia samples were enriched by density centrifugation over Ficoll-Paque and then washed twice in 10% RPMI-1640 medium supplemented with 2 mM L-glutamine (Sup-RPMI). For lymphoma biopsies, lymph nodes were surgically removed, immediately frozen in liquid nitrogen and kept at -80°C until further use (Department of Pathology, Hospital de Santa Maria, CHLN, Lisbon, Portugal). Upon diagnosis, we selected lymph nodes from lymphoma cases and reactive lymph nodes for our studies.

In vitro *killing assays*

For cytotoxicity assays, tumor cells (cell lines or primary samples) were stained with DDAO-SE (Molecular Probes, Invitrogen) and incubated at a ratio of 1:10 with γδ T cells in Sup-RPMI. Typically, 3×10^5 HMB-PP-activated γδ-PBL (>90% $V\gamma9^+$) were co-incubated with 3×10^4 tumor cells (pre-labeled with 1 μM DDAO-SE) for 3-4h, then stained with Annexin V-FITC (BD Biosciences) and analyzed by flow cytometry.

RNA isolation, RT-qPCR and Affymetrix Microarrays

Total RNA from tumor cell lines was extracted using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). RNA from leukemia cells and samples was extracted with TRIzol Reagent (Invitrogen) and purified with RNeasy Mini Kit according to the manufacturer's instructions. Concentration and purity was determined by spectrophotometry and integrity was confirmed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA, USA). Total RNA was reverse-transcribed into cDNA as previously described.[7] qPCR was performed on Rotor-Gene 6000 (Corbett) using SYBR Green detection system (PE Applied Biosystems). Glucoronidase beta (GUSB) and proteasome subunit beta type 6 (PSMB6) were used as endogenous controls in relative quantification using the standard curve method. Primers were designed using the Roche Design Centre (for sequences see *Online Supplementary Table S1*).

For genome-wide analyses, RNA from two independent cultures of each cell line (DAUDI, RAJI, RCH-ACV and 697) was processed for use on Affymetrix (Santa Clara, CA, USA) GeneChip HuGene 1.0 ST Arrays, according to the manufacturer's Whole Transcript Sense Target Labeling Assay.

Microarray data analysis

All the microarray data analysis was performed with R and several packages available from CRAN[17] and Bioconductor.[18] The raw data (CEL files) were normalized and summarized with the Robust MultiArray Average method from the "affy" package.[19] Unsupervised clustering analysis of the gene expression profiles for entire probe set data was assessed through hierarchical clustering (Euclidean distance and complete agglomeration method) and principal component analysis (prcomp function which calls a sin-

gular value decomposition method for non-symmetric matrices) as implemented in the statistical computing package.[17] Differentially expressed genes for each comparison were selected using linear models and empirical Bayes methods[20] as implemented in the Limma package,[21] verifying the *P* values corresponding to moderated F-statistics, and selecting as differentially expressed genes those that had adjusted *P* values adjusted using the Benjamini and Hochberg method[22] lower than 0.05.

The enrichment of biological functions and pathways was analyzed using Ingenuity Pathway Analysis software (Ingenuity Systems, Mountain View, CA, USA) and all genes present in the Affymetrix Human Gene 1.0 ST as control.

Results

Highly variable susceptibility of acute leukemias and non-Hodgkin's lymphomas to γδ-PBL cytotoxicity

In our laboratory we have studied a collection of 23 samples of acute lymphoblastic leukemias and non-Hodgkin's lymphomas, and a panel of 20 tumor cell lines of hematopoietic origin. The latter included acute lymphoblastic leukemia (ALL) (JURKAT, MOLT4, RCH-ACV, 697, CEM, TOM-1, RS4-11, B15, REH, Bv173) and acute myelogenous leukemia (AML) (HL-60, HEL, THP-1) cell lines; and non-Hodgkin Burkitt's (DAUDI, RAJI, RAMOS), follicular (DOHH2) and lymphoblastic (Oz) lymphoma cell lines (for detailed description of these cell lines see *Online Supplementary Table S2*). Although the capacity of peripheral blood γδ T cells to target multiple tumor cell lines of hematopoietic origin is well documented,[7-9] we observed that a substantial fraction of cell lines (Figure 1A and B) and patient samples (Figure 1A and *data not shown*) were strikingly resistant to γδ-PBL (obtained from healthy donors) pre-activated (as illustrated by high CD69 levels) with HMB-PP, the most potent natural Vγ9Vδ2 T-cell activator known to date (6, 7) (*Online Supplementary Figure S1*). For example, the B-ALL cell lines Bv173, REH and 697 (Figure 1 A and B), and six primary samples obtained from B-ALL patients (Figure 1A and *data not shown*) remained mostly alive (Annexin V-) in co-cultures with fully-activated (100% $CD69^+$; data not shown) γδ-PBL. Similar data were obtained with primary T-ALL samples and the cell line CEM (Figure 1A). This resistance to γδ-PBL cytotoxicity contrasted sharply with the extensive killing observed for the B-ALL line RCH-ACV and the T-ALL line MOLT-4 (Figure 1A), among various other hematopoietic tumors (Figure 1B).

For systematic analysis of our killing assay data, we considered tumor samples with over 70% lysis as susceptible to γδ-PBL-mediated lysis ("γδ-susceptible"), and those under 30% lysed as "γδ-resistant". Importantly, susceptibility was independent of the γδ-PBL donor, as the pattern of susceptible/resistant lines was equivalent for 3 independent healthy donors (*Online Supplementary Figure S2A*). Moreover, the differences in susceptibility to γδ T cells were maintained when tumor cell lines were incubated with γδ T cells activated for a shorter time (12h) (*Online Supplementary Figure S2B*), further supporting the segregation between susceptible and resistant cell

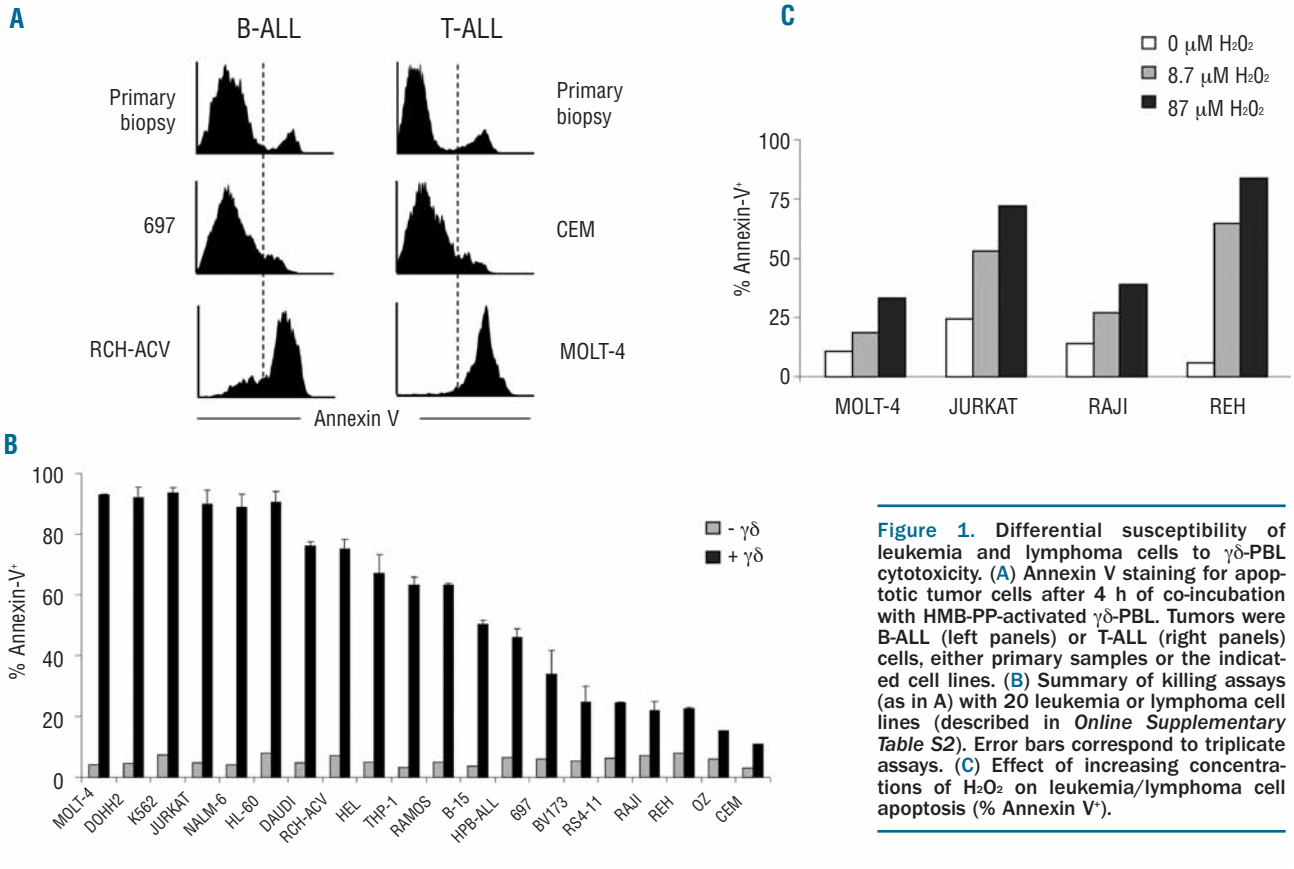

Figure 1. Differential susceptibility of leukemia and lymphoma cells to γδ-PBL cytotoxicity. (A) Annexin V staining for apoptotic tumor cells after 4 h of co-incubation with HMB-PP-activated γδ-PBL. Tumors were B-ALL (left panels) or T-ALL (right panels) cells, either primary samples or the indicated cell lines. (B) Summary of killing assays (as in A) with 20 leukemia or lymphoma cell lines (described in *Online Supplementary Table S2*). Error bars correspond to triplicate assays. (C) Effect of increasing concentrations of H_2O_2 on leukemia/lymphoma cell apoptosis (% Annexin V^+).

lines. As primary samples to reproduce and expand experiments aimed at dissecting the molecular mechanisms of tumor susceptibility to γδ-PBL cytotoxicity are difficult to obtain, we focused on our well-established panel of cell lines for the initial candidate searches and later extended our findings to patient samples.

We first considered that tumor resistance to γδ-PBL cytotoxicity could stem from intrinsic anti-apoptotic mechanisms developed by some leukemia/lymphoma cell lines. However, when we tested the effect of a pro-apoptotic stimulus (H_2O_2) we observed no association between resistance to apoptosis and to γδ-PBL cytotoxicity. Namely, the cell lines Jurkat (γδ-susceptible) and REH (γδ-resistant) were more sensitive to non-saturating concentrations of H_2O_2 than the cell lines MOLT-4 (γδ-susceptible) and RAJI (γδ-resistant) (Figure 1C). This suggests that susceptibility to γδ-PBL cytotoxicity is not related to the response to other death stimuli and probably involves a specific protein expression program (involved in tumor/γδ-PBL interactions) that we set out to characterize.

Genome-wide comparisons between γδ-susceptible and γδ-resistant hematopoietic tumors

The observed differences in susceptibility γδ-PBL cytotoxicity among hematopoietic tumors emphasize the importance of defining gene signatures that may predict the effectiveness of γδ T-cell based immunotherapies in the clinic. We performed a genome-wide analysis aimed at comparing the mRNA expression profiles of γδ-susceptible and γδ-resistant tumors. We employed cDNA microarrays to examine two pairs of hematopoietic tumor cell lines sharing the same cytogenetic alterations and cellular phenotypes (*Online Supplementary Table S2*): the Burkitt's lymphomas DAUDI (susceptible) and RAJI (resistant), and the B-ALL lines RCH-ACV (susceptible) and 697 (resistant).

First, samples were grouped according to the similarity of gene expression patterns using unsupervised clustering analysis (no group specification *a priori*). Based on the entire probe set data, two main groups could be defined which corresponded to the original cell type (Figure 2A): pre-B (697 and RCH-ACV) and mature B cells (DAUDI and RAJI). We next applied principal component analysis (PCA), which identifies new variables, to the principal components, which are linear combinations of the original variables (gene expression levels) and represent the largest variation found between samples.[23] Although the original cell type was the major source of variation between all samples (53.3% of total variation), PCA showed that component 3 was responsible for the segregation (16.4% of total variation) according to the susceptibility to γδ-PBL cytotoxicity (Figure 2B): susceptible (DAUDI and RCH-ACV) versus resistant (RAJI and 697).

To identify gene expression variations associated with susceptibility to γδ-PBL cytotoxicity, and to suppress the variations due to the transformed cell type (pre-B or mature B cells), we first compared tumors with identical origin, i.e. DAUDI *versus* RAJI, and RCH-ACV *versus* 697 (*Online Supplementary Tables S3 and S4*). We then used Bayesian linear models[20] and selected the common genes between both analyses: 340 genes (155 up- and 185 down-regulated in γδ-susceptible tumors) presented similar gene expression variations and were considered for further analysis (*Online Supplementary Table S5*). Bioinformatics analysis revealed an enrichment for functions related to cell-to-cell signaling and interaction, hematologic system development and function, immune

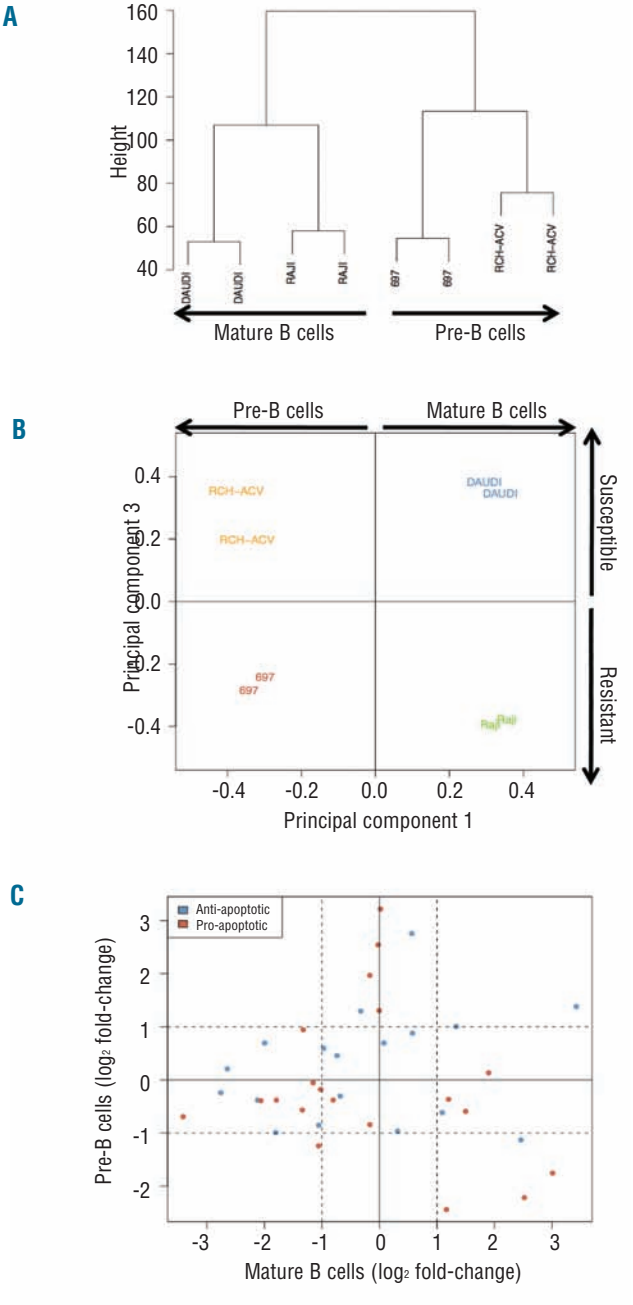

Figure 2. Comparison of gene expression in tumor cell lines susceptible or resistant to γδ-PBL cytotoxicity. Bioinformatics analyses of cDNA microarray comparisons between the Burkitt's lymphomas DAUDI and RAJI; and the B-ALL lines RCH-ACV and 697. (A) Unsupervised hierarchical clustering analysis. Samples with similar gene expression patterns are grouped together and connected with branches, producing a clustering tree (or dendrogram) on which the branch length inversely reflects the degree of similarity between samples. (B) Principal Component Analysis. The samples are plotted according to the first and third principal components (corresponding to the largest variation found between samples). (C) Variations in expression levels of anti- or pro-apoptotic genes in susceptible *versus* resistant tumor cell lines. Dashed lines indicate 2 fold-changes (in logarithmic scale) in the expression ratio susceptible/resistant.

cell trafficking (*P* value < 0.05; *Online Supplementary Table S6*). Some of the top pathways affected were interferon signaling, crosstalk between dendritic cells and natural killer cells, and molecular mechanisms of cancer (*P* value < 0.05; *Online Supplementary Table S7*).

The gene expression variations observed also suggested that, consistent with our previous experimental data (Figure 1C), the segregation between susceptible and resistant tumors is not associated with expression of anti- or pro-apoptotic genes (Figure 2C and *Online Supplementary Table S8*). Thus, up-/down-regulation of pro-/anti-apoptotic genes did not correlate with susceptibility to γδ-PBL cytotoxicity. Moreover, apoptotic related functions and pathways were not enriched in the panel of 340 genes (*Online Supplementary Table S7*). Based on these results, we favored the hypothesis that susceptibility or resistance to γδ-PBL cytotoxicity is conferred by signals presented at the tumor/γδ-PBL interface, i.e. on the surface of leukemia/lymphoma cells.

A set of cell surface proteins segregates between γδ-susceptible and γδ-resistant leukemia/lymphoma cell lines

T cells recognize their targets through cell surface antigens. We, therefore, focused our analysis of the panel of 340 genes on those encoding plasma membrane proteins (with extracellular domains), using a fold change threshold of 2 (log FC >1). These consisted of 8 genes up-regulated and 19 genes down-regulated in γδ-susceptible tumors when compared to resistant tumors (*Online Supplementary Table S9*). The mRNA expression levels of the 27 candidates were assessed by RT-qPCR (in independent samples) to validate the microarray results. Upon statistical analysis of the data, 22 out of the 27 genes were confirmed as differentially expressed in the two pairs of cell lines used for microarray comparisons: of these, 6 genes were up-regulated and 16 genes were down-regulated in γδ-susceptible tumors (Figure 3A). In order to have more stringent selection criteria, we extended our expression studies to a broader panel of cell lines, including 6 susceptible and 4 resistant cell lines (*Online Supplementary Figure S3*). This showed 10 genes with significant expression variation between susceptible and resistant tumors (*P* value < 0.05, Mann-Whitney test) (Figure 3B). Thus, our final panel of candidate markers of susceptibility to γδ-PBL cytotoxicity consisted of 3 genes enriched in γδ-suceptible tumors (*ULBP1, TFR2* and *IFITM1*), and 7 genes enriched in γδ-resistant leukemias/lymphomas (*CLEC2D, NRP2, SELL, PKD2, KCNK12, ITGA6* and *SLAMF1*) (Table 1).

Heterogeneity of expression of candidate markers in primary leukemia and lymphoma samples

We next determined the expression levels of each candidate marker in primary samples obtained from T-cell acute lymphoblastic leukemia (T-ALL) and non-Hodgkin's lymphoma (NHL) patients. Within the latter group, we sampled patients with common indolent (follicular) or aggressive (diffuse large B-cell - DLBCL) lymphomas. Gene expression levels in samples were compared with healthy PBMCs (for ALL) and reactive follicles (for NHL), taken as references (0 on log scale) in Figure 4A and B. Hence, a positive or negative (log scale) variation indicates higher or lower expression in tumors than in the control samples, respectively. Overall, the tumors exhibited very variable gene expression profiles. For example, among susceptibility-associated genes, *ULBP1* was over-expressed in a large number of primary samples, while *TFR2* was only enriched in three FL samples (FL 1, FL 2 and FL 8), and *IFITM1* was strongly depleted in various tumors (Figure 4A and B). On the other hand, all resistance-associated genes were over-expressed in FL sample 3, in contrast to the majority of primary samples analyzed. Moreover, there was no essential difference in some markers, such as ITGA6 or SELL, between the various patients (Figure 4A and B). Collectively, these data revealed a striking heterogeneity in the expression of particular candidate genes in primary tumors. When compared to our results with tumor cell lines (*Online Supplementary Figure S3*), these clinical data possibly reflect distinct selective pressures on the

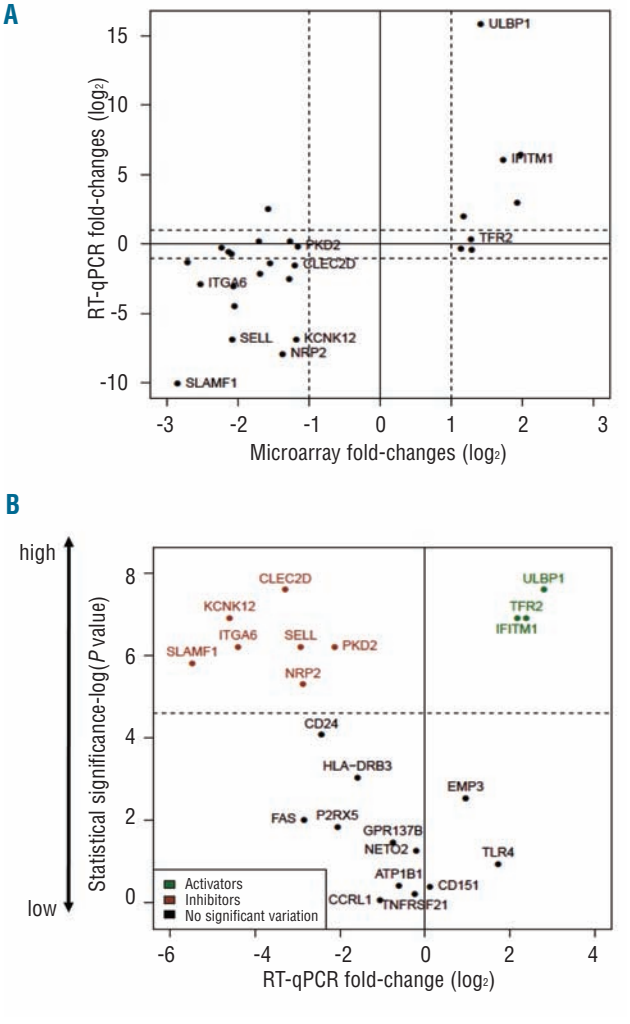

Figure 3. Variations in expression of genes encoding cell surface proteins that segregate between γδ-susceptible and γδ-resistant leukemia/lymphoma cell lines. (A) RT-qPCR validation of microarray results for the comparisons of Figure 2. The mRNA expression levels were normalized to GUSB and PSMB6 for each cell line. Plotted are the averages of relative expression levels in DAUDI versus RAJI (DAUDI/ RAJI) and RCH-ACV versus 697 (RCH-ACV/ 697). Dashed lines indicate 2 fold-change values (in logarithmic scale). (B) Statistical analysis of RT-qPCR results (detailed in Figure 4) in 6 susceptible and 4 resistant cell lines. Statistical significance was assessed by Mann-Whitney test (-log *P* value). Dashed line represents the statistical threshold *P*=0.01.

Table 1. Panel of cell surface proteins associated with the susceptibility or resistance of lymphomas/leukemias to γδ T-cell cytotoxicity. The statistical difference between the average gene expression in the 6 susceptible versus the 4 resistant tumors of Figure 4 was assessed by Mann-Whitney test ($P<0.05$).

Symbol	Description	Biological function	P value
Enriched in γδ-susceptible tumors			
ULBP1	UL16 binding protein	Ligand for NKG2D on NK and T cells; induces cytotoxicity, cytokine secretion	0
IFITM1 (CD225)	Interferon-induced transmembrane protein 1	Involved in cell proliferation and malignancy	0
TFR2	Transferrin receptor 2	Cellular uptake of transferrin-bound iron	0.004
Enriched in γδ-resistant tumors			
CLEC2D	C-type lectin 2, D	Ligand for the NK inhibitory receptor CD161	0.002
SELL	Selectin L	Adhesion of T cells to endothelial cells	0.001
SLAMF1	Signaling lymphocytic activation molecule 1	Bidirectional T cell to B cell stimulation	0
KCNK12	Potassium channel K, 12	Potassium channel	0
ITGA6	Integrin alpha 6	Integrin; receptor for laminin	0.014
PKD2	Polycystic kidney disease 2	Calcium channel	0.017
NRP2	Neuropilin 2	Co-receptor for VEGF; implicated in tumor growth and vascularization	0.018

expression of the genes that compose the candidate panel, the consequence of which should now be evaluated in clinical trials.

Discussion

The success of immunotherapy to tackle tumors, in particular those that prevail after chemo- or radiotherapy, critically depends on two factors: the specific activation of effector anti-tumor lymphocytes and the molecular recognition of tumor cells by activated lymphocytes. Concerning γδ T cells, research over the last 15 years has identified very potent and specific phosphoantigens, most notably HMB-PP,[6,7] that seem to fulfill the first requirement. There have been suggestions that phosphoantigens themselves,[6,24,25] or an F1-ATPase-related structure complexed with delipidated apolipoprotein A-I,[26] or the non-classical MHC protein ULBP4[27] could be responsible for tumor cell recognition by Vγ9Vδ2 PBL. However, despite this, the issue is still highly controversial. This naturally impacts on our ability to design effective therapeutic protocols based on γδ-PBL immunotargeting of tumors. Thus, only 33% of patients with prostate carcinoma[11] or non-Hodgkin's lymphoma[14] showed objective responses despite large activation and expansion of their Vγ9Vδ2 T cells *in vivo*. These considerations stress the importance of identifying tumor molecular signatures that may predict the response to activated γδ-PBL.

In this study, we set out to identify cell surface proteins involved in interactions between leukemia/lymphoma cells and γδ-PBL. Taking *in vitro* tumor cytolysis as functional readout, we screened a panel of 20 leukemia and lymphoma cell lines that faithfully reproduced the susceptibility/resistance of primary tumors (Figure 1A). The use of cell lines permitted experimental reproducibility and hence statistical robustness for the gene expression undertaken. Upon the identification of candidate markers, we analyzed their expression in 23 samples derived from T-ALL and NHL (FL and DLBCL) patients.

The choice of cDNA microarrays as screening tools was based on a multiplicity of previous studies that demonstrated how powerful and reliable they are in defining cancer molecular signatures.[28] Our analyses led to the identification of a large panel of genes differentially expressed between "γδ-susceptible" and "γδ-resistant" tumors. Importantly, we verified that there was no correlation between intrinsic anti-apoptotic properties and resistance to γδ-PBL cytotoxicity, both in terms of gene expression and response to a death stimulus. Thus, susceptibility or resistance to γδ-mediated lysis is more likely to be related to tumor recognition and immune evasion strategies, the molecular basis of which remains to be clarified. Of note, MHC class Ia expression did not consistently segregate between γδ-susceptible and γδ-resistant tumor cell lines (*Online Supplementary Figure S4*). For example, among susceptible lines, DAUDI and MOLT-4 expressed very low or undetectable levels, whereas JURKAT and RCH-ACV displayed high levels of surface MHC class I (*Online Supplementary Figure S4*). These data exclude a mechanism of "missing self" as the basis for γδ T-cell recognition of hematopoietic tumors.

Building upon stringent biological and statistical selection criteria, we narrowed our microarray data down to 10 genes encoding cell surface proteins (with extracellular domains), whose expression segregated with susceptibility *versus* resistance to γδ-PBL cytotoxicity. We believe it is important to make this gene profile available to the biomedical community. Thus, we propose the expression of each candidate gene to be evaluated during upcoming γδ T-cell based clinical trials. The genes with highest predictive value will constitute novel leukemia/lymphoma biomarkers, for which standardized quantification essays should be developed. This will provide clinicians with a key tool for the indication and monitoring of γδ T-cell based immunotherapies.

Furthermore, within the panel of 10 candidate markers, some are likely to play non-redundant roles in leukemia/lymphoma cell recognition by γδ-PBL. Thus, proteins that are enriched in γδ-susceptible tumors may provide activation signals, whereas markers of resistance may convey inhibitory signals to γδ-PBL. Provocatively, 7

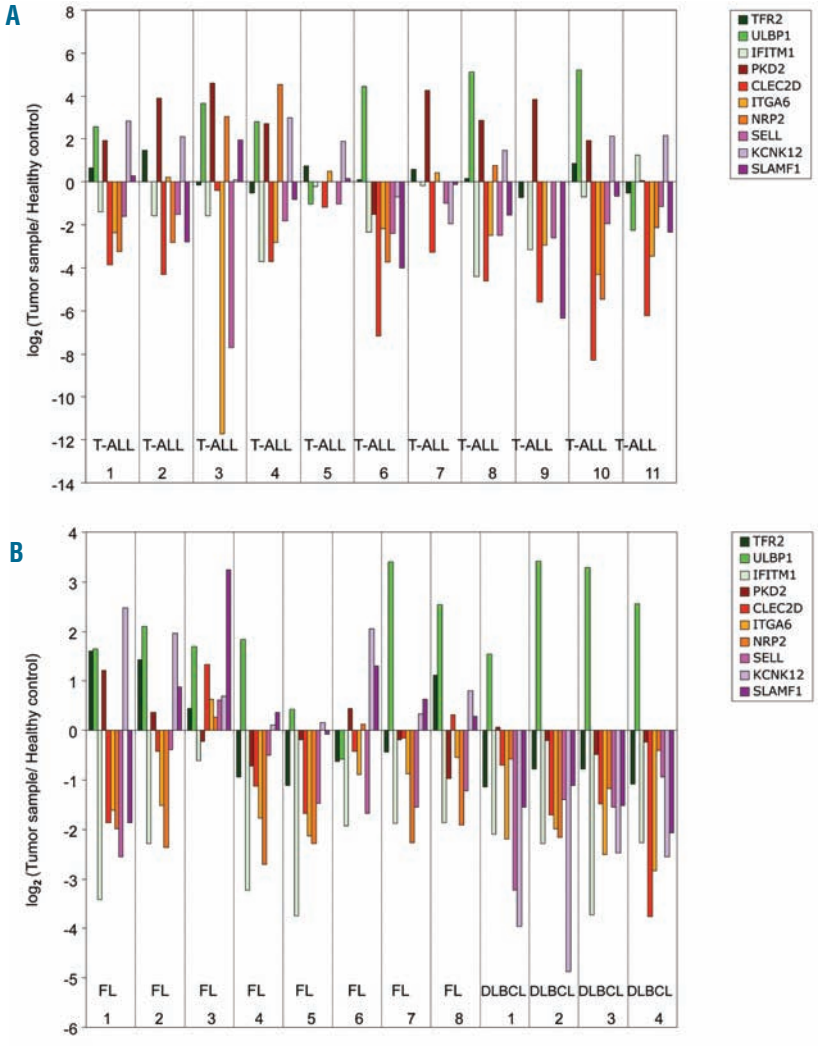

Figure 4. Quantification of mRNA expression levels of γδ-susceptibility markers in acute lymphoblastic leukemia and non-Hodgkin's lymphoma patients. (A) RT-qPCR analysis of mRNA expression in 11 T-cell acute lymphoblastic leukemia (T-ALL) samples, normalized to housekeeping genes (*GUSB* and PSMB6) and to reference PBMCs from healthy individuals. Values were converted to logarithmic scale. (B) RT-qPCR analysis of mRNA expression in 8 follicular lymphoma (FL) and 4 diffuse large B cell lymphoma (DLBCL) samples, normalized to housekeeping genes (*GUSB* and *PSMB6*) and to a reference sample - reactive follicles – obtained through the same procedure. Values were converted to logarithmic scale.

of the candidates are known to intervene in immune responses: 4 of them (ULBP1, IFITM1, CLEC2D and SLAMF1) provide stimulatory (or inhibitory) signals through receptors expressed on lymphocytes, while 3 (NRP2, SELL and ITGA6) control lymphocyte adhesion. ULBP1 is a ligand for the NKG2D receptor expressed on all cytotoxic lymphocyte lineages, including 100% of Vγ9Vδ2 T cells, which has been clearly implicated in anti-tumor responses.[29-32] IFITM1 was shown to modulate NK cell responses and its expression correlated with improved survival of gastric cancer patients.[33] By contrast, the expression of CLEC2D, a ligand for the inhibitory receptor CD161, inhibits NK cell responses and was associated with increased malignancy grade of glioblastoma.[34] NRP2 is another protein that can favor cancer progression by acting as a coreceptor for vascular endothelial growth factor (VEGF) and stimulating tumor growth (35). We will now proceed with individual knock-down (RNA interference) experiments in a functional (tumor killing) bioassay to dissect the role of each of the candidates in γδ-PBL targeting of leukemias and lymphomas. Given that some of these molecules can also provide costimulatory or inhibitory signals to NK cells, we also plan to address their role in NK cell targeting of hematopoietic malignancies.

In summary, this report establishes a panel of 10 putative markers of leukemia/lymphoma susceptibility to γδ-PBL cytotoxicity. The expression data collected from primary samples showed a striking heterogeneity for particular candidate genes, most notably *ULBP1*, whereas other genes, such as *IFITM1*, *ITGA6* or *SELL*, essentially did not vary among patients. It is, therefore, predictable that different components of the proposed panel will behave in very distinct ways when associated to therapeutic outcome in clinical trials. It will also be interesting to evaluate to what extent immunoselection may have conditioned the expression of these markers in tumors evolving in a dynamic interaction with γδ T lymphocytes. This will significantly add to our understanding of anti-tumor immunity and to our capacity to modulate it for cancer immunotherapy.

Authorship and Disclosures

BSS was the principal investigator and takes primary responsibility for the paper. AQG, DVC and TL performed the laboratory work for this study. ARG performed the bioinformatics analysis of the data. CF, JFL, JTB and MGS provided clinical samples and suggestions. AQG, DVC and BSS wrote the manuscript.

The authors reported no potential conflicts of interest.

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