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Low frequency of V γ 9V δ 2 T cells predicts poor survival in newly diagnosed acute myeloid leukemia

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Key Points

- A low frequency of V γ 9V δ 2 T cells in AML is an independent predictor of poor clinical outcome.
- These data provide a strong rationale for the development of consolidation protocols aimed at enhancing V γ 9V δ 2 T-cell responses.

In several tumor subtypes, an increased infiltration of V γ 9V δ 2 T cells has been shown to have the highest prognostic value compared with other immune subsets. In acute myeloid leukemia (AML), similar findings have been based solely on the inference of transcriptomic data and have not been assessed with respect to confounding factors. This study aimed at determining, by immunophenotypic analysis (flow or mass cytometry) of peripheral blood from patients with AML at diagnosis, the prognostic impact of V γ 9V δ 2 T-cell frequency. This was adjusted for potential confounders (age at diagnosis, disease status, European LeukemiaNet classification, leukocytosis, and allogeneic hematopoietic stem cell transplantation as a time-dependent covariate). The cohort was composed of 198 patients with newly diagnosed (ND) AML. By univariate analysis, patients with lower V γ 9V δ 2 T cells at diagnosis had significantly lower 5-year overall and relapse-free survivals. These results were confirmed in multivariate analysis (hazard ratio [HR], 1.55 [95% confidence interval (CI), 1.04-2.30]; $P = .030$ and HR, 1.64 [95% CI, 1.06-2.53]; $P = .025$). Immunophenotypic alterations observed in patients with lower V γ 9V δ 2 T cells included a loss of some cytotoxic V γ 9V δ 2 T-cell subsets and a decreased expression of butyrophilin 3A on the surface of blasts. Samples expanded regardless of their V γ 9V δ 2 T-cell levels and displayed similar effector functions in vitro. This study confirms the prognostic value of elevated V γ 9V δ 2 T cells among lymphocytes in patients with ND AML. These results provide a strong rationale to consider consolidation protocols aiming at enhancing V γ 9V δ 2 T-cell responses.

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The data sets used and/or analyzed during this study are available on reasonable request from the corresponding author, Daniel Olive (daniel.olive@inserm.fr).

The full-text version of this article contains a data supplement.

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Introduction

The immune landscape of acute myeloid leukemia (AML) is becoming better described, and there is increasing evidence that immune features may modulate prognosis independently of genetic markers.¹⁻³ However, immune alterations in AML are broadly heterogeneous among patients in terms of immune infiltration and expression of immune checkpoint receptors,⁴⁻⁶ suggesting distinct immunoregulatory mechanisms and complicating the development of immune signatures.^{4,7} This complexity also led to a slow clinical translation of immunotherapeutic strategies in AML.^{3,8} Most studies have so far focused on conventional T cells^{2,5,9} or natural killer (NK) cells,¹⁰⁻¹² but very little data are available on the impact of $\gamma\delta$ T-cell populations in AML.

The major role of $\gamma\delta$ T cells has emerged in infectious diseases and antitumoral immunity.¹³ These cells combine adaptive and innate characteristics. Their activation is not restricted by the major histocompatibility complex^{14,15} and depends on ligand recognition by their T-cell receptor (TCR) and other receptors such as DNAX accessory molecule-1 (DNAM-1)¹⁶ or natural killer group 2 member D (NKG2D).¹⁷ These unique recognition capabilities distinguish them from other lymphocytes. Human $\gamma\delta$ T cells represent 1% to 10% of circulating T cells.¹⁸ Based on the TCR δ -chain, they can be classified into 4 major groups: V δ 1, V δ 2, V δ 3, and V δ 5. V δ 2⁺ is the major subtype in peripheral blood (PB) and usually pairs with the V γ 9 chain. V δ 1⁺ accounts for 25% of PB $\gamma\delta$ T cells and V δ 3⁺ for <1%.¹⁹ A direct rapid effector response is exhibited by $\gamma\delta$ T cells, which have the ability to activate other immune cells through cytokine release or through their antigen presentation capabilities.^{20,21}

The progressive discovery of the elusive mechanism of action of $\gamma\delta$ T cells and of their prognostic implications has evolved in parallel with $\gamma\delta$ T-cell-based immunotherapy approaches, with promising results. The latter include allogeneic adoptive transfer of $\gamma\delta$ T cells, from $\alpha\beta$ T-cell-depleted products, and of ex vivo expanded $\gamma\delta$ T-cell infusions, with optimized expansion strategies or genetic engineering.²² Adoptive transfer is currently at the forefront in AML, several studies evaluating the efficacy of ex vivo expanded allogeneic $\gamma\delta$ T cells in refractory or relapsed (R/R) AML. In vivo stimulation strategies targeting V γ 9V δ 2 T cells were initially based on the use of amino-biphosphonates but have shown limited efficacy.²³ However, new methods of autologous V γ 9V δ 2 T-cell-based immunotherapy are emerging, in particular with bispecific or monoclonal antibodies (mAbs) targeting $\gamma\delta$ TCRs directed against tumoral antigens such as human epidermal growth factor receptor 2 (HER-2)²⁴ or CD123.²⁵

V γ 9V δ 2 T cells are activated by phosphoantigens (pAgs), produced by infected or transformed cells, via the mevalonate pathway during isoprenoid biosynthesis.^{26,27} The antitumoral activity of V γ 9V δ 2 T cells is triggered by pAgs binding to the intracellular domain of butyrophilins (BTNs) 3A1 and 2A1.²⁸⁻³² V γ 9V δ 2 T cells exert pleiotropic antitumoral potential against a wide range of tumors, including hematological malignancies.³³ A particular activity of V γ 9V δ 2 in AML is suspected because they exhibit a high cytotoxic potential against myeloid blasts both in vitro and in vivo.³⁴⁻³⁸ Early clinical trials of V γ 9V δ 2 T-cell-based immunotherapy have indeed led to objective responses in AML.^{39,40} The frequency of V γ 9V δ 2 T cells has also been observed to vary according to induction chemotherapy responses.^{41,42}

Importantly, analysis of nearly 18 000 bulk transcriptomes from 39 human tumors has revealed the high prognostic value of high $\gamma\delta$ T-cell infiltration, which had the best prognostic value compared with other immune subsets.⁴³ Another study confirmed the association between V γ 9V δ 2 T-cell infiltration and favorable outcomes in a broad spectrum of cancers.⁴⁴ Nevertheless, deconvolution of bulk transcriptomes using the CIBERSORT algorithm raises the issue of misclassification for some immune subsets. Besides, conflicting data have been reported with a negative role of $\gamma\delta$ T cells in several solid tumors,⁴⁵⁻⁴⁷ possibly based on protumoral properties for some $\gamma\delta$ T-cell subsets depending on the tumoral context.⁴⁸

In AML, demonstration of the poor prognostic impact of a low frequency of V γ 9V δ 2 T cells at diagnosis is based on results extrapolated from bulk RNA sequencing analysis.⁴⁴ Yet, the presence of potential confounding factors has not been assessed, preventing any solid conclusion on this matter.

Therefore, the aim of this study was to determine the prognostic impact of the PB frequency of V γ 9V δ 2 T cells, determined by immunophenotypic analysis, in newly diagnosed (ND) treatment-naïve patients with AML, adjusting for potential confounding factors.

Methods

Study design

This study merged 4 different cohorts: 2 from the Hematology Department of Institut Paoli-Calmettes (IPC), 1 retrospective (IPC tumor bank, IPC16-007; n = 21) and 1 prospective (HEMATO-BIO-IPC 2013-015, NCT02320656, ANSM 131368B-11; n = 40); 2 cohorts from prospective multicenter randomized phase 3 trials from the French Innovative Leukemia Organization (FILO) group, AML-2006-IR cohort (NCT00860639; n = 84) and LAM-SA 2007 cohort (NCT00590837; n = 53). Patients from the IPC cohort were diagnosed between December 2002 and October 2018 and FILO patients between November 2007 and November 2012. PB mononuclear cells (PBMcs) from healthy volunteers (HVs) were obtained from the Etablissement Français du Sang (authorization number AC-2019-3428 issued by the Ministry of Higher Education, Research and Innovation).

Patients were aged from 18 to 81 years and were treated with conventional anthracycline-based induction chemotherapy. In the AML-2006-IR cohort, patients were then randomly assigned to receive a single dose of gemtuzumab ozogamicin in arm A. In the LAM-SA 2007 cohort, patients were randomized to receive lomustine or not during the induction and postinduction treatment phases.

Patients were included if PB V γ 9V δ 2 T-cell frequency could be assessed.

Patients with AML3, with unknown or adverse European LeukemiaNet (ELN) risk classification were excluded. Patient characteristics are summarized in Table 1.

AML diagnosis was established according to the World Health Organization classification.⁴⁹ Cytogenetic and molecular analyses were used for patient stratification. Response to treatment and relapse were evaluated according to ELN 2010 recommendations.⁵⁰

Table 1. Baseline patient and disease characteristics

	All patients N = 198
Age, y	
Median (range)	56.0 (18.9-81.5)
>60, n (%)	75 (37.9)
Sex, n (%)	
Male	109 (55.05)
Female	89 (44.95)
AML etiology, n (%)	
De novo	185 (93.4)
Secondary	13 (6.6)
WBC, ×10⁹/L	
Median (range)	25.7 (0.6-191.3)
>50, n (%)	56 (28.3)
NA, n (%)	2 (1.0)
FAB category, n (%)	
M0	4 (2.0)
M1	46 (23.2)
M2	43 (21.7)
M3	0 (0)
M4	51 (25.8)
M5	35 (17.7)
M6	4 (2.0)
M7	0 (0)
NA	15 (7.6)
PB blasts, %	
Mean (SD)	50.4 (32.9)
ELN classification, n (%)	
Favorable	79 (39.9)
Intermediate	119 (60.1)
CBF mutation, n (%)	
24 (12.1)	
CEBPA mutation, n (%)	
20 (13.5)	
NPM1 mutation, n (%)	
92 (53.8)	
FLT3 mutation, n (%)	
58 (34.1)	
Vγ9Vδ2 (% of LC)	
Mean (SD)	2.5 (4.3)
30-d mortality, n (%)	3 (1.5)
60-d mortality, n (%)	11 (5.6)
CR after induction, n (%)	161 (81.3)
HSCT, n (%)	32 (16.3)

CBF, core-binding factor; CEBPA, CCAAT enhancer binding protein alpha; FAB, French-American-British classification; FLT3, fms-like tyrosine kinase 3; LC, lymphocyte; NPM1, nucleophosmin 1; WBC, white blood cell; SD, standard deviation.

All participants provided written informed consent in accordance with the Declaration of Helsinki.

Clinical samples

PBMCs obtained before induction chemotherapy or from HV were isolated by density gradient (Lymphoprep, Stemcell Technologies,

Vancouver, Canada) and cryopreserved in 90% fetal calf serum/10% dimethyl sulfoxide (DMSO) (FILO), 90% albumin/10% DMSO (HEMATOBIO and age-matched HV), or 80% RPMI/ or 10% fetal calf serum/10% DMSO (IPC tumor bank).

Sample handling, conditioning, and storage were respectively performed by the tumor banks of the FILO group (N° BB-0033-00073) and the IPC tumor bank (AC-2007-33 granted by the French Ministry of Research).

Flow and mass cytometry analysis

Samples were included if at least 1 000 000 viable cells could be labeled. The proportion of V γ 9V δ 2 T cells among PB lymphocytes was assessed by flow or mass cytometry. Supplemental Tables 2 and 3 provide the list of mAbs used, and supplemental Figure 9 describes the gating strategy.

For flow cytometry, PMBCs were washed and incubated with human Fc Block (BD Biosciences, San Jose, CA) before immunostaining with a mix of extracellular antibodies (supplemental Table 2). A fluorescence-activated cell sorter (FACS) LSR-Fortessa or a BD FACSCanto II (BD Biosciences) were used for acquisition, and data were analyzed using DIVA 8.0.1 software (BD Biosciences).

The mass cytometry experiments were performed as previously described.⁵¹ Extracellular and intracellular antibodies are listed in supplemental Table 3. Cells were acquired on a Helios mass cytometer (Fluidigm), and samples were further analyzed using FlowJo V10.6.2 (BD Biosciences). Statistical analyses of markers expressed by V γ 9V δ 2 T cells were performed when >30 or >50 viable V γ 9V δ 2 T cells were analyzed in mass or flow cytometric cytometry panels, respectively.

V γ 9V δ 2 T cells were manually gated and exported using FlowJo V10.6.2. Consensus files were generated using the OMIQ software from Dotmatics with a downsampling to a total number of 10 000 V γ 9V δ 2 T cells for each group. V γ 9V δ 2 T-cell subpopulations and markers were described using the t-distributed stochastic neighbor embedding (t-SNE) dimensionality reduction algorithm.

Generation of anti-human BTN3A mAbs

To generate anti-BTN3A 20.1 and anti-BTN3A 108.5 mAbs, BALB/c mice were immunized with a soluble BT3.1-Ig fusion protein, as previously described.⁵² The relative surface expression of BTN3A (mAbs 20.1 and 108.5) on AML blasts was then assessed by flow cytometry using calibrator beads, allowing for the quantification of antigen density (Quantum Simply Cellular, Bangs Laboratories, Fishers, IN). Cells were acquired on a BD FACS-Canto II and analyzed using Flowjo V10.6.2.

Expansion of V γ 9V δ 2 T cells

For the establishment of allogeneic V γ 9V δ 2 T cells, fresh PBMCs from HV were stimulated with zoledronate (ZOL) 1 μ M (Sigma-Aldrich, Saint Louis, MO) and recombinant human interleukin-2 (rhIL-2; Miltenyi Biotec, Bergisch Gladbach, Germany) at day 0. From day 5, rhIL-2 (100 IU/mL) was renewed every 2 days, and cells were kept at 1.5 × 10⁶/mL until day 14. Only cell cultures that reached >80% of V γ 9V δ 2 T-cell purity on day 14 were selected and frozen until use. Allogeneic V γ 9V δ 2 T cells were thawed and cultured with rhIL-2 (200 UI/mL) overnight before functional assays.

For the establishment of autologous V γ 9V δ 2 T cells, thawed PBMCs from patients with AML were treated similarly, but rIL-15 (10 ng/mL) was also added to enhance their proliferative capacities.⁵³⁻⁵⁵ Fresh autologous expanded cells were then used for functional assays, depending on the quantity of V γ 9V δ 2 T cells. Fold increases of viable V γ 9V δ 2 T cells were calculated according to the following formula: (day-14 %V γ 9V δ 2 \times day-14 total cell number)/(day-0 %V γ 9V δ 2 \times day-0 total cell number). Cells were acquired on a BD FACSCanto II and analyzed using DIVA 8.0.1 software.

Degranulation assays

To analyze CD107 expression, autologous V γ 9V δ 2 T cells and primary AML blasts were cocultured in an effector-to-target ratio of 1:1 with anti-CD107a, anti-CD107b, GolgiStop, and anti-BTN3A 20.1 agonist mAb or isotype control (1 μ g/mL). Primary AML blasts preincubated overnight with zoledronate (45 μ M) were used as positive control. After 4 hours, cells were collected and analyzed by flow cytometry. Cells were acquired on a BD FACSCanto II and analyzed using DIVA 8.0.1 software.

Statistics

Statistical analyses were carried out using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA), Statistical Package for the Social Sciences (SPSS software, Chicago, IL), and R software (www.rproject.org).

Normality of distributions was assessed using the d'Agostino-Pearson normality test.

Comparisons of categorical variables were performed using χ^2 or Fisher exact tests. For continuous variables, comparisons between 2 groups used 2-tailed Mann-Whitney *U* test or unpaired *t* test. For multiple comparisons of paired values, a Friedman test was performed followed by a Dunn's test. For multiple comparisons of independent samples, a Kruskal-Wallis test was performed followed by a Dunn's test. Patient groups were defined according to the frequency of V γ 9V δ 2 T cells among lymphocytes. The threshold was defined based on optimized cutoff using maximally selected log-rank statistics (maxstat package, R software V 3.6.2).⁵⁶

A multivariate Cox regression model was used to assess the prognostic value of V γ 9V δ 2 T-cell frequency, adjusting for other prognostic factors: age at diagnosis, disease status, ELN classification, and allogeneic hematopoietic stem cell transplantation (HSCT) as time-dependent covariate.

Results

Patients with AML with a low frequency of V γ 9V δ 2 T cells have poorer survival

A total of 198 patients were included. Their mean age was 56 years (range, 18.9-81.5); 79 (39.9%) had favorable ELN risk and 119 (60.1%) an intermediate ELN risk. The mean complete remission (CR) rate was 81.3% (Table 1). Analysis of overall survival (OS) and relapse-free survival (RFS), based on the frequency of V γ 9V δ 2 T cells, showed the 0.75% threshold to be the most significantly discriminant (Figure 1A-B) and identified the high and low frequencies referred to thereupon.

As summarized in Table 2, patients with AML with lower V γ 9V δ 2 T cells tended to be older (56.9 vs 54.5 years; *P* = .058). No difference was observed in V γ 9V δ 2 T-cell frequency group according to the French-American-British classification,⁴⁹ response to induction in all cohorts (Table 1; supplemental Figure 1A); however, patients with favorable ELN risk had a lower V γ 9V δ 2 T-cell frequency (supplemental Figure 1B). In the FILO cohorts, patients who received lomustine in the LAM-SA 2007 trial (*n* = 21) were more often in the low V γ 9V δ 2 T cells group (supplemental Table 1). As V γ 9V δ 2 T cells are also critical effectors against infection,⁵⁷ the proportion of infectious complications was compared after induction in the FILO cohorts depending on V γ 9V δ 2 T cells, but no difference was observed in terms of incidence of septic shock nor G3/4 infectious adverse events.

In univariate analysis, patients with a low frequency of V γ 9V δ 2 T cells had significantly lower 5-year OS and 5-year RFS (43.1% vs 64%; hazard ratio [HR], 1.54 [95% confidence interval (CI), 1.03-2.31]; *P* = .028 and 42.3% vs 67.2%; HR, 1.69 [95% CI, 1.09-2.62]; *P* = .015, respectively). These results were confirmed in multivariate analyses (HR, 1.55 [95% CI, 1.04-2.30]; *P* = .030 and HR, 1.64 [95% CI, 1.06, 2.53]; *P* = .025; Table 3). CR and early mortality rates were similar in both groups of V γ 9V δ 2 T-cell frequency (Table 2).

Relapsed patients were found to have a significantly lower frequency of V γ 9V δ 2 T cells than those with sustained CR (supplemental Figure 2A). However, the proportions of NK cells and other lymphocyte subsets were similar regardless of outcome in the FILO cohorts (supplemental Figure 2B-C), as well as CD4⁺ and CD8⁺ T cells in the HEMATOBIO cohort (supplemental Figure 2D-E). Additionally, V γ 9V δ 2 T cells were found to contribute the most to the prognostic effect of $\gamma\delta$ T cells, suggesting that V δ 1 subtype predominance is not involved (supplemental Figure 2F-I).

Interestingly, the impact of a low frequency of V γ 9V δ 2 T cells was independent of ELN risk (supplemental Figure 3), allogeneic HSCT (supplemental Figure 4), or age (supplemental Figure 5).

Hence, a low frequency of V γ 9V δ 2 T cells was independently associated with poor outcomes. This adverse impact was likely related to an increased risk of relapse, as evidenced by the similar CR or early mortality rates.

Low V γ 9V δ 2 T-cell levels are associated with immunophenotypic shifts of the V γ 9V δ 2 T-cell compartment

The immunophenotype of V γ 9V δ 2 T cells was then examined according to their frequency. In the FILO cohorts, we observed a great heterogeneity in the expression of V γ 9V δ 2 T-cell coactivating receptors, with a significant decrease in the expression of NKG2A, CD57, and DNAM-1 in patients with low V γ 9V δ 2 T-cell frequencies. At variance, there was no difference in NKG2D or CD56 levels (Figure 2A). Moreover, no association was observed between the expression of these 5 molecules and clinical outcomes (supplemental Figure 6).

In mass cytometry, a much broader panel was used to detect the various differentiation states and cytotoxic T-cell subsets in a

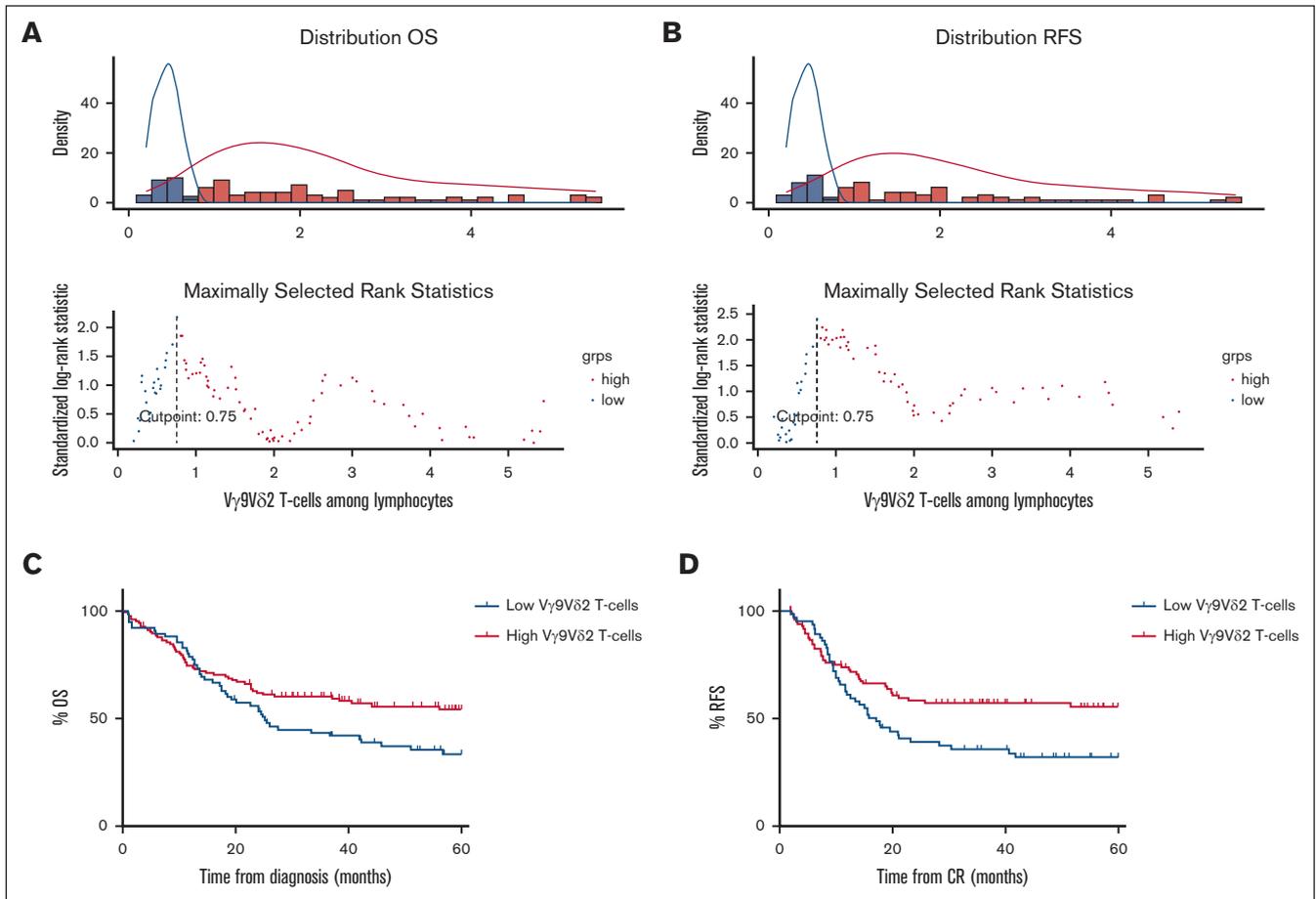


Figure 1. Univariate survival analysis according to the frequency of V γ 9V δ 2 T cells. (A-B) Threshold determination of V γ 9V δ 2 T cells among viable lymphocytes is determined using the optimal cut points, performed using the maximally selected log-rank statistics using the maxstat R package. For a given prognostic parameter, maxstat identifies the optimal cut point that best discriminates 2 patient groups (<https://cran.r-project.org/web/packages/maxstat/index.html>). The threshold of 0.75 % of V γ 9V δ 2 T cells among viable lymphocytes was selected for this study. (C-D) 5-y OS and 5-y RFS (n = 198 and 161, respectively) were estimated using the Kaplan-Meier method and compared between groups using the log-rank test.

subgroup of patients from the HEMATOBIO cohort and HVs. This disclosed no difference between patients with a high frequency of V γ 9V δ 2 T cells and HVs (Figure 2B-H). In this cohort, we confirm decreased levels of NKG2A and DNAM-1 in patients with low V γ 9V δ 2 T-cell frequencies (Figure 2C). Patients with low V γ 9V δ 2 T-cell frequencies expressed lower levels of CD69 and CD16 than HVs and patients with high V γ 9V δ 2 T-cell frequencies (Figure 2D), indicating a less activated TCR state and a lower antibody-dependent cell cytotoxicity potential.⁵⁸ In contrast to the FILO cohorts, we observed higher CD56 levels and similar CD57 expression in patients with low V γ 9V δ 2 T-cell frequencies. These discrepancies are relative, because CD56 and CD57 levels followed the same trend in both cohorts, and could be related to the heterogeneity of these markers on V γ 9V δ 2 T cells.⁵⁹⁻⁶¹ We did not find any differences in terms of maturation states (Figure 2E) or expression of triggering or coinhibiting molecules (Figure 2F-H), apart from a lower expression of inducible T-cell costimulator (ICOS) in patients with low V γ 9V δ 2 T-cell frequencies than HVs and patients with high V γ 9V δ 2 T-cell frequencies.

Analysis of surface ligand expression on the surface of AML blasts disclosed that patients with fewer V γ 9V δ 2 T cells had a lower expression of the BTN3A molecule (Figure 2I-J).

Altogether, these findings revealed that a “low V γ 9V δ 2 T-cell profile” is related to a loss of mature and cytotoxic subsets. This finding could explain the poor prognostic influence of a low frequency of V γ 9V δ 2 T cells, related to a loss of the most immunocompetent V γ 9V δ 2 T cells. This also suggests an important role for BTN3A in modulating the maturation and the proliferation of V γ 9V δ 2 T cells.

The nonproliferating low V γ 9V δ 2 T-cell immunophenotype can be reversed

The expansion capacities of V γ 9V δ 2 T cells were then explored with respect to their frequency. Using ZOL-stimulation combined to IL-2 plus IL-15 for 14 days, V γ 9V δ 2 T cells from PBMCs of 43 patients with AML at diagnosis were expanded. Cells from patients with a lower frequency of V γ 9V δ 2 T cells tended to expand better (low vs high V γ 9V δ 2 T cells; $P = .072$; Figure 3A).

Table 2. Baseline patient and disease characteristics according to the frequency of V γ 9V δ 2 T cells

	Low V γ 9V δ 2 T cells ($\leq 0.75\%/LC$)	High V γ 9V δ 2 T cells ($> 0.75\%/LC$)	P value
	n = 75 (37.9%)	n = 123 (62.1%)	
Age, y			
Median (range)	56.9 (22.4-80.4)	54.5 (18.9-81.5)	.058
>60	30 (40)	45 (36.6)	.652
Sex, n (%)			
Male	38 (50.7)	71 (57.7)	.378
Female	37 (49.3)	52 (42.3)	
AML etiology, n (%)			
De novo	69 (92)	116 (94.3)	.562
Secondary	6 (8)	7 (5.7)	
WBC, $\times 10^9/L$			
Median (range)	30.2 (0.6-191.3)	24.6 (1.1-154.9)	.451
>50, n (%)	23 (31.1)	33 (26.8)	.423
NA, n (%)	1 (1.3)	1 (0.8)	
FAB category, n (%)			
M0	2 (2.7)	2 (1.6)	.397
M1	15 (20.0)	31 (25.2)	
M2	14 (18.7)	29 (23.6)	
M3	0 (0)	0 (0)	
M4	22 (29.3)	29 (23.6)	
M5	16 (21.3)	19 (15.4)	
M6	0 (0)	4 (3.3)	
M7	0 (0)	0 (0)	
NA	6 (8.0)	9 (7.3)	
PB blasts, %			
Mean (SD)	49.5 (31.1)	50.9 (34.1)	.828
ELN classification, n (%)			
Favorable	33 (44.0)	46 (37.4)	.373
Intermediate	42 (56.0)	77 (62.6)	
CBF mutation, n (%)	10 (13.3)	14 (11.4)	.823
CEBPA mutation, n (%)	7 (13.0)	13 (13.8)	>.999
NPM1 mutation, n (%)	37 (56.9)	55 (51.9)	.532
FLT3 mutation, n (%)	22 (34.4)	36 (34.0)	>.999
Vγ9Vδ2 T cells among LC, %			
Mean (SD)	0.4 (0.2)	3.9 (5.1)	<.0001
30-d mortality, n (%)	1 (1.3)	2 (1.6)	>.999
60-d mortality, n (%)	6 (8.0)	5 (4.1)	.339
CR after induction, n (%)	64 (85.3)	97 (78.9)	.347
HSCT, n (%)	12 (16.0)	20 (16.5)	>.999

CBF, core-binding factor; CEBPA, CCAAT enhancer binding protein alpha; FAB, French-American-British; FLT3, fms-like tyrosine kinase 3; LC, lymphocyte; NPM1, nucleophosmin 1; WBC, white blood cell; SD, standard deviation.

Some of the samples were also functionally tested by measuring the degranulation of V γ 9V δ 2 T cells. Surprisingly, there was no difference in degranulation capacities between V γ 9V δ 2 T-cell frequency groups whatever the tested conditions. As previously described by our team, pAg-stimulation resulted in an increased degranulation of expanded autologous V γ 9V δ 2 T cells.³⁴

Furthermore, BTN3A targeting with the 20.1 agonist mAb led to an increase in degranulation compared with controls.

This demonstrated that the use of an anti-BTN3A agonists empower degranulation capacities of autologous V γ 9V δ 2 T cells against primary AML blasts, confirming results that had only been

Table 3. Univariate and multivariate analysis of factors influencing survival

Variable	5-y OS						5-y RFS					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value	HR	95% CI	P value
Age, y												
>60 vs ≤60	2.21	(1.46-3.34)	<.0001	1.90	(1.27-2.83)	.002	1.98	(1.25-3.13)	.001	1.57	(1.01-2.46)	.045
WBC, ×10⁹/L												
>50 vs ≤50	0.64	(0.4-1.01)	.093	NE*			0.99	(0.6-1.59)	.977	NE*		
ELN classification												
Intermediate vs favorable	1.80	(1.22-2.67)	.005	1.95	(1.26-3.00)	.003	1.77	(1.15-2.71)	.012	2.04	(1.28-3.25)	.003
Status												
Secondary vs de novo	1.35	(0.62-2.92)	.393	NE*			1.67	(0.71-3.92)	.142	1.21	(0.59-2.47)	.595
Vγ9Vδ2 T cells among LC, %												
≤0.75 vs >0.75	1.54	(1.03-2.31)	.028	1.55	(1.04-2.30)	.030	1.69	(1.09-2.62)	.015	1.64	(1.06-2.53)	.025
HSCT												
Yes vs no	0.46	(0.28-0.76)	.017	0.59	(0.30-1.16)	.127	0.53	(0.31-0.88)	.045	0.38	(0.17-0.86)	.019

5-y OS and 5-y RFS were estimated using the Kaplan-Meier method and compared between groups using the log-rank test (n = 198 and 161, respectively). Among the usual AML prognostic factors (age, WBC, ELN 2010 risk category, de novo vs secondary AML, and HSCT), those with a P value <.15 were included in the multivariate logistic regression, with HSCT as a time-dependent covariate (n = 196 and 159 respectively for 5-y OS and 5-y RFS).

LC, lymphocyte; NE, not entered; WBC, white blood cell.

*Not entered in the model.

obtained by using allogeneic V γ 9V δ 2 T cells expanded from HVs.³⁶ Interestingly, it was also observed that ages of patients with AML did not influence the ability of V γ 9V δ 2 T cells to expand and degranulate (supplemental Figure 7).

Overall, these expansion and degranulation experiments demonstrated that the functional impact of the immunophenotype associated with a low frequency of V γ 9V δ 2 T cells was reversible in vitro, even in older patients.

Discussion

Probably because of their major histocompatibility complex-unrestricted mechanism of activation, the prognostic impact of $\gamma\delta$ T cells in AML has been mostly studied after HSCT. Here, a reduced frequency of circulating V γ 9V δ 2 T cells at AML diagnosis was shown to be independently associated with worse outcome.

These findings support results suggesting the prognostic role of V γ 9V δ 2 T-cell abundance assessed by deconvolution of bulk tumor transcriptomes using algorithms such as CIBERSORT-LM22⁴³ or CIBERSORT-LM7.⁴⁴ However, such approaches based on bulk transcriptomic data carry a risk of erroneous results, especially when the analysis is focused on such a rare immune subset as that of V γ 9V δ 2 T cells, in particular at AML diagnosis. In addition, transcriptome analysis is difficult to apply in clinical routine. Moreover, the presence of confounding factors had not been tested in these studies, thus preventing solid conclusions regarding the prognostic value of PB V γ 9V δ 2 T-cell frequency in AML.

The results reported here are also consistent with studies evaluating the good prognostic impact of reconstitution of a high frequency of $\gamma\delta$ T cells on OS and RFS after HSCT.⁶²⁻⁶⁴ However, the distinction between V δ 2⁺ and other $\gamma\delta$ T-cell subsets had only been occasionally analyzed, and the prognostic value of this reconstitution in AML remained controversial.^{64,65}

In contrast to the many immunomonitoring studies available after HSCT,⁶⁶ immunophenotypic approaches to evaluate V γ 9V δ 2 T cells at diagnosis have been limited to very small cohorts^{42,67} and evidenced no impact of V γ 9V δ 2 T-cell immunophenotype according to clinical outcomes. It was shown that the PB frequency of V γ 9V δ 2 T cells varies according to the quality of the response to induction⁴¹ and is lower after induction in R/R patients than patients in sustained CR.⁴² By contrast, in the study reported here, patients with lower V γ 9V δ 2 T cells at diagnosis responded similarly to induction but had a higher risk of relapse. Therefore, it could be hypothesized that the frequency of V γ 9V δ 2 T cells at diagnosis rather represents their proliferative capabilities, which could be further inhibited in R/R patients.

Importantly, a decreased frequency of V γ 9V δ 2 T cells was associated with pronounced immunophenotypic alterations that affect predominantly a decreased expression of cytotoxicity and/or activation markers, including NKG2A, CD57, DNAM-1, and CD16. Our results are consistent with previous natural killer receptor blockade assays of autologous V γ 9V δ 2 T cells from patients with AML that showed that masking of DNAM-1 decreased the cytotoxic functions of V γ 9V δ 2 T cells against autologous blasts, whereas masking of NKG2D did not.³⁴ In addition, CD16 plays a key role in effector functions of V γ 9V δ 2 T-cell functions,^{58,68} including against acute lymphoblastic leukemia or chronic lymphocytic leukemia cells.^{69,70} Meanwhile, downregulation of NKG2A was also demonstrated on V γ 9V δ 2 T cells from patients with AML with lower V γ 9V δ 2 T cells, and NKG2A has recently been reported to characterize a subset of “educated” circulating V γ 9V δ 2 T cells that exert great antitumor functions.⁷¹ CD57 is thought to be a marker of replicative senescence in $\alpha\beta$ T cells,⁷² but its significance in V γ 9V δ 2 T cells is debated.⁷³ Indeed, CD57⁺ V γ 9V δ 2 T cells proliferate less⁷⁴ but do not accumulate with age.⁵⁹⁻⁶¹ Regarding coinhibitory molecules, a low frequency of V γ 9V δ 2 T cells was only associated with an

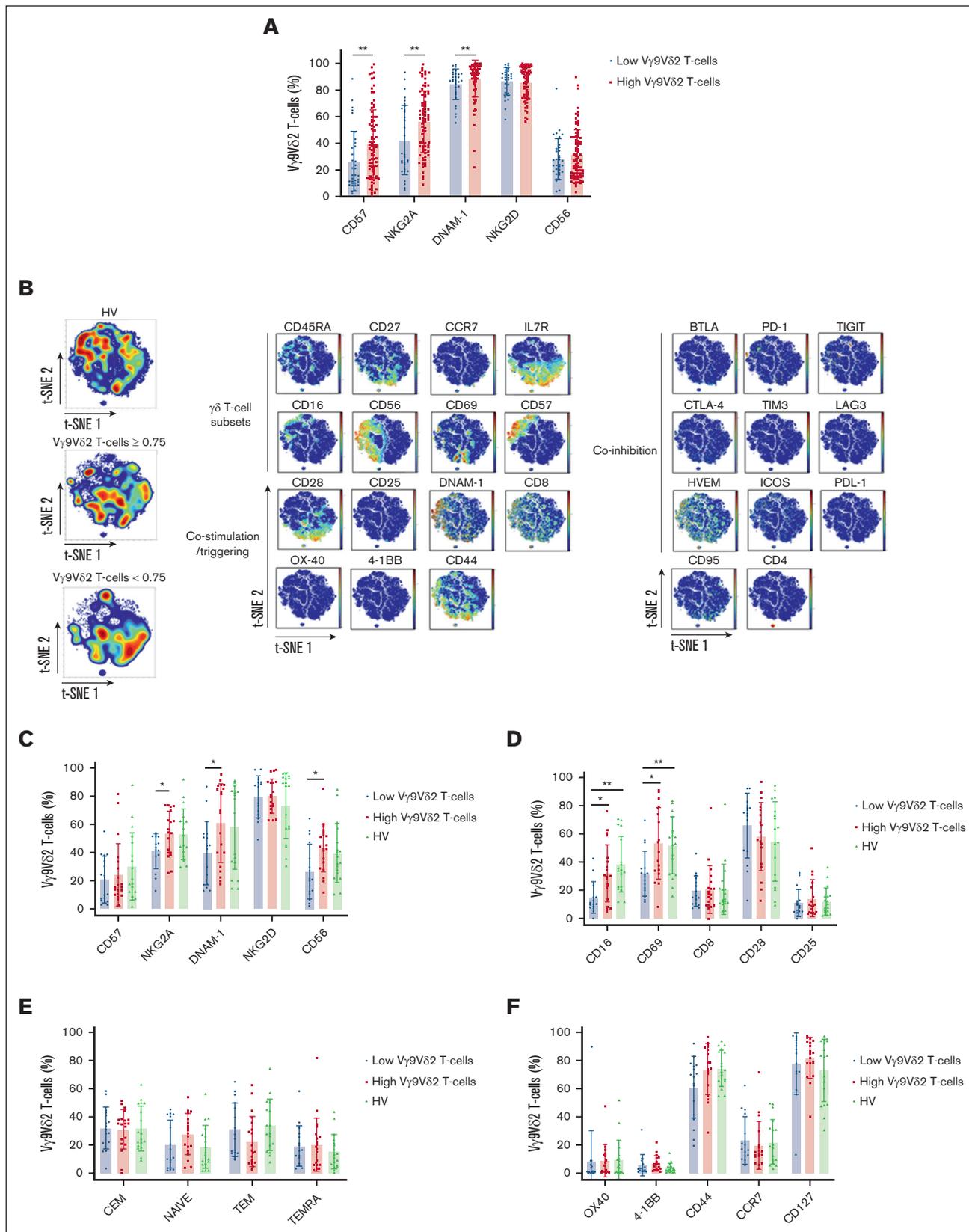


Figure 2. Low V γ 9V δ 2 T-cell phenotype is associated with phenotypical changes. (A) Expression of surface V γ 9V δ 2 T-cell coreceptors in the FILO cohorts according to the frequency group of V γ 9V δ 2 T cells: percentage of cells positive for CD57 (n = 123), NKG2A (n = 116), DNAM-1 (n = 116), NKG2D (n = 115), and CD56 (n = 123). (B) V γ 9V δ 2 T cells from PBMCs of HVs and patients with AML from the HEMATOBIIO cohort were manually pre-gated and exported in OMIQ for t-SNE analysis. Subsampling was

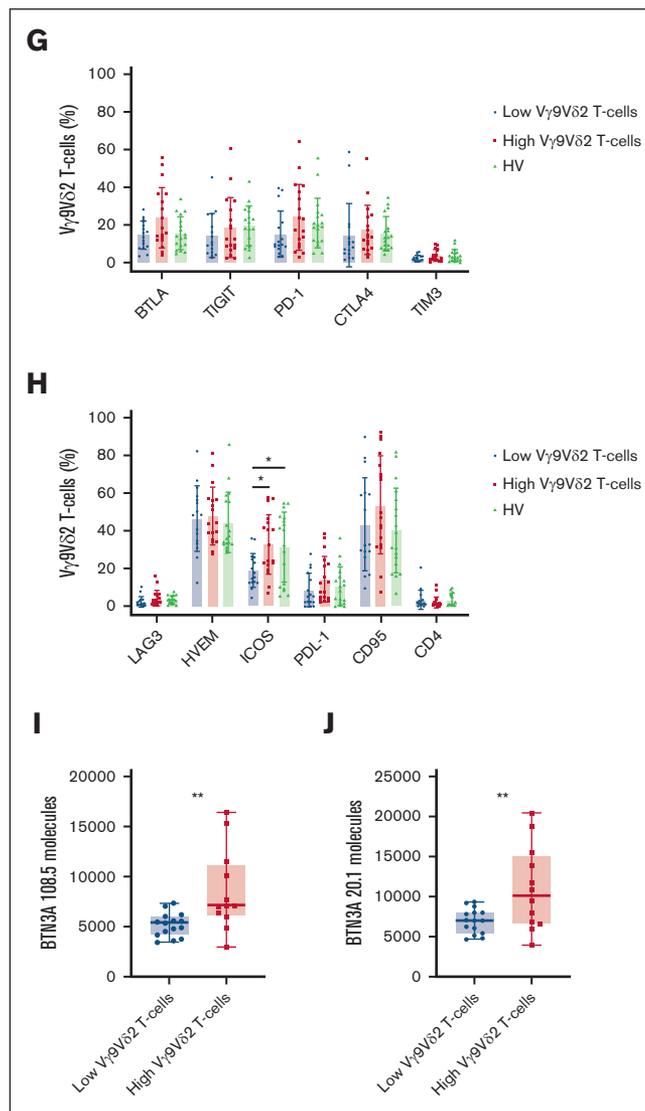


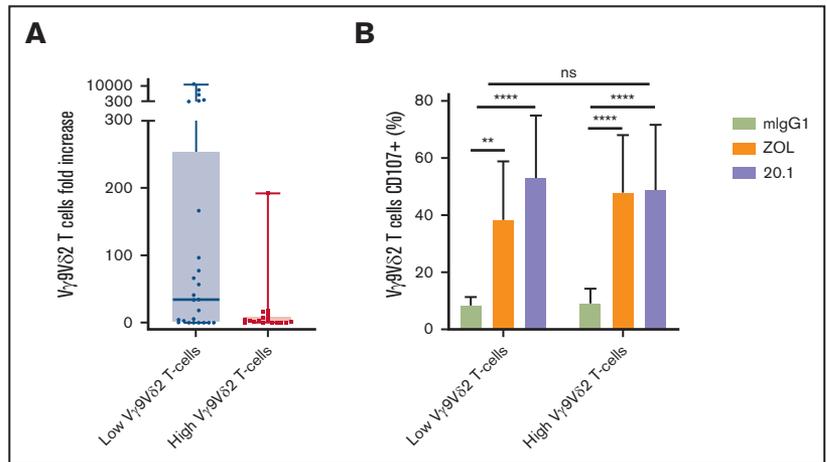
Figure 2 (continued) performed for HVs and for each patient group with a fixed number of 100 000 V γ 9V δ 2 T cells for each group (HV, n = 8; patients with AML with low V γ 9V δ 2 T cells, n = 12; patients with AML with high V γ 9V δ 2 T cells, n = 7). V γ 9V δ 2 T cells were analyzed using t-SNE dimensionality reduction algorithm. In the left panel, the density of V γ 9V δ 2 T-cell subsets in each patient group is projected (blue, low cell density; red, high cell density). The expression of markers of V γ 9V δ 2 T cells is projected onto t-SNE maps in the right panel (blue, low expression; red, high expression). (C-H) Maturation phenotype and expression of surface V γ 9V δ 2 T-cell coreceptors from PBMCs of HVs (n = 18) and patients with AML from the HEMATOBIIO cohort (n = 40) according to the V γ 9V δ 2 T-cell frequency group (n = 20 in each group): (C) percentage of V γ 9V δ 2 T cells positive for NKG2A, CD57, DNAM-1, NKG2D, and CD56; (D) percentage of V γ 9V δ 2 T cells positive for CD16, CD69, CD8, CD28, and CD25; (E) maturation phenotype of V γ 9V δ 2 T cells according to the expression of CD45RA and CD27; (F) percentage of V γ 9V δ 2 T cells positive for OX40, 4-1BB, CD44, CCR7, and CD127; (G) percentage of V γ 9V δ 2 T cells positive for BTLA, TIGIT, PD-1, CTLA4, and TIM3; (H) percentage of V γ 9V δ 2 T cells positive for LAG3, HVEM, ICOS, PDL-1, CD95, and CD4. (I-J) BTN3A expression was assessed by flow cytometry on the surface of primary blasts from patients with AML (n = 27), the relative number of BTN3A molecules was quantified with MESF (molecules of equivalent soluble fluorochrome) method, using the anti-BTN3A 108.5 (I) and 20.1 epitopes (J). Results are presented as mean \pm standard deviation (SD) (A, C-H) or as box plots (I-J), and statistical significance was established using a Mann-Whitney test (A), or a Kruskal-Wallis test followed by a Dunn's test (C-H), or a *t* test (I-J). **P* < .5; ***P* < .01.

upregulation of ICOS on V γ 9V δ 2 T cells, suggesting that these V γ 9V δ 2 T cells are neither exhausted nor senescent at diagnosis, in contrast to the signature of terminal senescence often observed in $\alpha\beta$ T cells of patients with ND AML.⁴² Interestingly, ICOS/ICOS-ligand (ICOS-L) pathway has been involved in NK-cell activation

by pAg-activated V γ 9V δ 2 T cells and may lead to the acquisition of DC editing function by NK cells.⁷⁵

However, further investigations are needed to understand the respective qualitative^{42,67,76} vs quantitative^{41,43,44} contribution of

Figure 3. Autologous expansion rates and functional capacities according to V γ 9V δ 2 T-cell phenotype. (A) PBMCs from patients with AML (n = 43) were cultured with 1 μ M zoledronate (ZOL) plus 200 U/mL rhIL-2 and 10 μ g/mL rhIL-15 for 14 days. (B) Degranulation of V γ 9V δ 2 T cells expanded from PBMCs of patients with AML (n = 23) against autologous blasts pretreated or not with ZOL 50 μ M and with or without the agonist anti-BTN3A 20.1 mAb. Results are presented as box plots (A) or as mean \pm SD (B), and the statistical significance was established using a Mann-Whitney test (A-B) or a Friedman test followed by a Dunn's test (B). ***P* < .01; *****P* < .0001.



V γ 9V δ 2 T cells in ND AML, as well as the associated alterations in other immune subtypes and their dynamic changes over the course of treatment. Interestingly, although phenotypically altered and/or nonproliferating, V γ 9V δ 2 T cells retained their ability to kill autologous blasts, as we have recently shown in acute lymphoblastic leukemia.⁷⁷ These data confirm the persistent functional plasticity of V γ 9V δ 2 T cells leading to an adaptive response in leukemia.

Finally, it was shown here that surface BTN3A levels on AML blasts could influence the frequency of V γ 9V δ 2 T cells. We were limited by the number of cells needed to perform other coculture assays, but it could be hypothesized that more BTN3A molecules on the blast surface might make them more “visible” to V γ 9V δ 2 T cells, with a subsequent impact on their proliferation. This is consistent with a recent transcriptomic study showing a positive association between $\gamma\delta$ T-cell infiltration and BTN3A expression in head and neck squamous cell carcinoma.⁷⁸ Alternatively, V γ 9V δ 2 T-cell proliferation could be inhibited by a specific mechanism such as defective mevalonate metabolism in AML blasts³⁸ or by more general immune escape processes such as enhanced inhibitory ligand expression, reduced expression, or shedding of activating ligands.³ For instance, baseline plasmatic levels of soluble BTN3A and BTN2A1 have been associated with outcomes in several solid tumors.⁷⁹⁻⁸¹ The underlying mechanisms of BTN3A level expression are still unknown but have been investigated in a recent CRISPR screen that showed a multilayered regulation dependent on a metabolic crisis in tumor cells,⁸² and further studies are warranted to understand BTN3A-specific regulation in AML blasts. In addition, targeting BTN3A on AML blasts improved the cytotoxic functions of autologous V γ 9V δ 2 T cells, demonstrating that BTN3A could be a reliable target to “awaken” V γ 9V δ 2 T cells from patients with AML who have decreased frequency of this subset. These observations warrant further investigation of the efficacy of anti-BTN3A agonist antibodies in AML, such as ICT01, which is currently being evaluated in the multicenter phase 1/2 EVICTION trial.⁸³

Age-related immune alterations in AML are closely associated with poor clinical outcomes with a higher proportion of late effector T cells,⁴ limiting the individualization of an independent effect of immune alterations in older patients with AML. In the present cohorts, age-related changes appeared to be similar to the

previously described frequency of circulating V γ 9V δ 2 T cells in older patients.⁸⁴⁻⁸⁶ Interestingly, a low PB V γ 9V δ 2 T-cell frequency was found to yield a similar prognostic impact whatever the age of the patients. Moreover, older patients had similar expansion and degranulation capacities as younger ones, indicating a persistent V γ 9V δ 2 T-cell plasticity with aging, as already described in HVs.^{60,74} Similarly, it was confirmed that V γ 9V δ 2 T cells adopt a more resilient behavior and are more resistant to senescence than most immune effectors.^{60,74,85,87} Collectively, these results demonstrate that V γ 9V δ 2 T-cell levels retain a prognostic impact in older patients, and immunotherapeutic approaches to harness their functions in this population could be developed to reduce the risk of relapse. In this regard, recent findings suggest that the use of 5-azacitidine or venetoclax may potentiate the antileukemic functions of V γ 9V δ 2 T cells, as demonstrated by encouraging results in combination with ICT01.⁸⁸ It would be also interesting to evaluate the prognostic contribution of V γ 9V δ 2 T cells in a cohort of patients with AML treated with hypomethylating agents and/or venetoclax.

In summary, evidence is reported here that a low PB frequency of V γ 9V δ 2 T cells in ND AML is independently associated with poor prognosis. Although this reduced frequency of V γ 9V δ 2 T cells is associated with major phenotypic alterations, the remaining V γ 9V δ 2 T cells can proliferate and exert cytotoxic functions against autologous blasts. Altogether, these findings confirm that V γ 9V δ 2 T cells act as critical stress-sensors in AML and provide a strong rationale for harnessing and potentiating these immune effectors in the early treatment of AML.

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Authorship

Contribution: A.-C.L.F., A.-S.C., and D.O. conceptualized and designed the study; A.-C.L.F., F.O., A.B.A., M.-S.R., N.S., C.C., C.D., A.-S.C., and A.L.R. performed experiments and interpreted the data; N.V. treated patients and coordinated the study; J.-F.H.,

N.I., P.C.-L., J.D., and E.D. coordinated the research; A.-C.L.F. and D.O. coordinated the research and wrote the manuscript; and all authors critically reviewed the manuscript.

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