

Expression of CD226 on $\gamma\delta$ T cells is lower in advanced chronic lymphocytic leukemia and correlates with IgA, IgG and LDH levels

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Abstract

Background. Gamma-delta ($\gamma\delta$) T cells comprise an important subset of human T cells, responding to viral and bacterial infections, and are significant for cancer immunosurveillance. Human $\gamma\delta$ T cells are divided into 5 major subsets, namely V δ 1–V δ 5, of which the latter 3 have limited available literature. At present, V δ 2 is the most studied subpopulation.

Objectives. In the current paper, we focused on non-V δ 2 cells in chronic lymphocytic leukemia (CLL). We assessed the expression of co-inhibitory checkpoint receptors (CTLA-4, PD-1 and TIGIT) and co-stimulatory (CD226 and NKp30) molecules separately on V δ 1 and V δ 3–V δ 5 cells.

Materials and methods. We assessed $\gamma\delta$ T cells for their expression of both cytotoxicity-related (NKp30, CD226) and co-inhibitory (PD-1, TIGIT) molecules with flow cytometry in CLL patients. Moreover, we evaluated the expression of TIGIT and CD226 ligand (PVR, CD155) in neoplastic B cells in CLL patients with quantitative real-time polymerase chain reaction (qPCR).

Results. A significant accumulation of V δ 1 T cells was noted, while no difference was observed in the total percentage of V δ 2 cells. Contrary to our initial hypothesis, the impact of CLL burden on CD226 and TIGIT expression was lower than anticipated. The former tends to be lower in more advanced disease. Finally, a strong upregulation of CD155 (PVR) was noted on CLL-derived B cells when compared to healthy B cells.

Conclusions. Chronic lymphocytic leukemia regulates the expression of the CD155–CD226/TIGIT axis. Contrary to expectations, the ligand is significantly more affected than the receptors. Nevertheless, the relatively high expression of CD155 and TIGIT makes CLL an interesting target for anti-TIGIT immunotherapy.

Key words: CLL, V δ 1, chronic lymphocytic leukemia, V δ 3–V δ 5, $\gamma\delta$ T

Cite as

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Background

Chronic lymphocytic leukemia (CLL) is an indolent leukemia derived from mature B cells.¹ It is mainly diagnosed in older adults and is the most common adult leukemia.^{2,3} However, despite significant progress, it remains incurable.⁴ Thus, patients are commonly observed, and treatment is initiated only after certain conditions are fulfilled.⁴ Furthermore, CLL is associated with a high tumor burden as neoplastic B cells can easily comprise >90% of the total peripheral blood mononuclear cells.⁵ This leads to the creation of a significant immunosuppressive environment and pushes T cells towards exhaustion.^{6,7} Exhausted T cells show alterations in their cytokine secretion profiles, low proliferative rates and, most importantly, overexpression of multiple inhibitory receptors on their surfaces (e.g., PD-1, Lag-3, Tim-3, or TIGIT).⁸ Notably, T cell exhaustion is comprehensively described for $\alpha\beta$ T lymphocytes and the V δ 2 subset of human $\gamma\delta$ T cells.⁹

Gamma-delta T cells constitute a major subset of unconventional T cells and are characterized by the expression of TCR γ and δ chains instead of α and β chains. Murine $\gamma\delta$ T cells are usually split up into subsets by their V γ fragment, while human $\gamma\delta$ T cells can be divided based on V δ fragment into V δ 1–V δ 5.^{10,11} V δ 2 cells comprise the major subset of circulating $\gamma\delta$ T cells and can be easily expanded in vitro with aminobisphosphonates. Therefore, the majority of $\gamma\delta$ T cell research is oriented towards the V δ 2 subtype.^{12,13} V δ 1 cells constitute the 2nd largest portion of $\gamma\delta$ T cells in peripheral blood, but due to more complicated protocols for their expansion, they are far less understood.¹⁴ V δ 3, V δ 4 and V δ 5 are virtually unstudied, mostly due to the lack of appropriate monoclonal antibodies that enable easy immunophenotyping or sorting.

Gamma-delta T cells are severely affected by the CLL disease burden.¹³ However, current data suggest that this applies unequally to different subsets. While V δ 2 cells show signs of exhaustion and are usually almost unresponsive, V δ 1 cells remain highly active in a large portion of CLL patients.^{9,15} Indeed, expansion and domination of V δ 1

may have an important positive influence on the course of CLL.^{13,15} While the clinical-grade large-scale expansion of autologous V δ 2 lymphocytes currently seems unreachable, there are such attempts with V δ 1 cells.¹⁴ Nevertheless, successful immunotherapy with V δ 1 cells requires a better understanding of the impact that CLL has on both $\gamma\delta$ T cells as a whole and their non-V δ 2 compartment.

Objectives

The current paper presents the results of immunophenotyping of human non-V δ 2 cells in CLL patients. The study was based on the hypothesis that the immunosuppressive CLL environment would upregulate the expression of co-inhibitory molecules while downregulating the co-stimulatory potential. Finally, we demonstrated that non-V δ 2 cells seem to be mildly affected by CLL, at least in terms of co-inhibitory and co-stimulatory molecule expression.

Materials and methods

Patients and material

A total of 37 treatment-naïve patients diagnosed with CLL were recruited from the Department of Hematology and Bone Marrow Transplantation at the Medical University of Lublin (Poland). Disease staging was performed according to the Rai classification.^{4,16} An age- and sex-matched control group was recruited from the Department of Interventional Radiology and Neuroradiology at the Medical University of Lublin and consisted of 20 individuals. Peripheral blood mononuclear cells (PBMCs) were obtained by gradient centrifugation in LSM 1077 (PromoCell, Heidelberg, Germany). Clinically important data were obtained from hospital records, including gene deletions in *ATM* (11q22.3 locus) and *TP53* (17p13.1 locus). Table 1 summarizes the basic characteristics of patients and controls.

Table 1. Characteristics of patients and controls

Characteristics	Patients	Controls
Number of patients	37	20
Percentage of men	53.38%	61.11%
Age, mean (\pm SD)	64.46 (\pm 10.73)	65 (\pm 10.58)
Percentage of ZAP-70-positive patients*	23.68%	–
Percentage of CD38 ⁺	26.32%	–
Percentage of patients with del(17p13.1) or del(11q22.3)	21.62%	–
Percentage of patients with Rai stage 0 (low-risk)	18.91%	–
Percentage of patients with Rai stages I–II (intermediate-risk)	43.24%	–
Percentage of patients with Rai stages III–IV (high-risk)	37.84%	–

* Leukemic B cells (CD5⁺/CD19⁺) were considered positive for ZAP-70 or CD38 expression with a cutoff point of \geq 20%. SD – standard deviation.

Immunophenotyping

Peripheral blood mononuclear cells were incubated with a mix of monoclonal antibodies consisting of anti-CD3 APC (BioLegend, San Diego, USA; cat. No. 300439, clone: UCHT1), anti-TCR $\gamma\delta$ BV421 (BD Biosciences, Warsaw, Poland; cat. No. 744870, clone: 11F2), anti-V δ 1 FITC (ThermoFisher, Warsaw, Poland; cat. No. TCR2730, clone: TS8.2), anti-V δ 2 PE (BioLegend; cat. No. 331408, clone: B6), anti-CTLA-4 APC-Fire750 (BioLegend; cat. No. 349930, clone: L3D10), anti-PD-1 PE-Cy7 (BD Biosciences, Franklin Lakes, USA; cat. No. 561272, clone: EH12.1), anti-TIGIT BV650 (BD Biosciences; cat. No. 747840, clone: 741182), anti-NKp30 BV785 (BioLegend; cat. No. 325230, clone: P30-15), and anti-CD226 BV605 (BD Biosciences; cat. No. 742495, clone: DX11). Fluorescence minus one (FMO) controls were used to set the correct gates for each fluorophore. Samples were acquired on a Cytoflex LX (Beckman Coulter, Warsaw, Poland) and analyzed with FlowJo 10 (BD Biosciences). The percentage of positive cells was a standard measure for each of the markers except for CD226, where mean fluorescence intensity (MFI) was additionally provided due to its high overall expression.

Bioinformatics

Available RNAseq datasets were used to assess the expression of CD226/TIGIT ligands on the surface of neoplastic B cells. The Chronic Lymphocytic Leukemia (Broad, Nature 2015) dataset was queried on cBioPortal.^{17–19} The expression of *PVR*, *NECTIN2* and *NECTIN3* was assessed in a subset of 157 CLL patients with mRNA expression data. Expression in transcripts-per-million (TPM) was extracted.

qPCR

B cells were isolated magnetically from healthy donors and CLL patients with anti-CD19 microbeads and LS columns (Miltenyi Biotec, Bergisch Gladbach, Germany). Total RNA was isolated with a Blood RNA Mini kit (Qiagen, Hilden, Germany) and reverse transcription was performed with QuantiTect Reverse Transcription Kit (Qiagen). Finally, quantitative real-time polymerase chain reaction (qPCR) was performed with qPCR Master Mix (Promega, Madison, USA) and primers for *PVR* and *GAPDH*. Reactions were run with the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Austin, USA). Gene expression was normalized to *GAPDH* and is presented as 2^{- Δ CT}. Primer sequences:

PVR for.: 5' GGATATCTGGCTCCGAGTGC 3'

PVR rev.: 5' CTCCACCTTGCAGGTCACAT 3'

GAPDH for.: 5' ATCACCATCTTCCAGGAGCG 3'

GAPDH rev.: 5' TGGACTCCACGACGTACTCA 3'

Statistical analyses

Results were analyzed with GraphPad Prism v. 9 (GraphPad Software, San Diego, USA) and JASP 0.16.3 (Department of Psychological Methods, University of Amsterdam, Netherlands). A Shapiro–Wilk test was used for data distribution analysis. The p-values were calculated with Mann–Whitney U tests, and differences were considered significant when $p < 0.05$. Data are presented as the median and interquartile range (IQR). Correlations were assessed with a Spearman's test. In the statistical analysis, we employed an exploratory approach, not including a correction for families of hypotheses, which leads to an increased risk of type 1 error. Thus, the results must be interpreted with caution. The study might serve as a starting point for further research in the field. Detailed p-values for each comparison are presented in Supplementary Table 1.

Results

V δ 1 cells are expanded in CLL patients

First, we assessed the percentage of V δ 1 and V δ 2 T cells. While the percentage of V δ 2 cells seemed unaffected by CLL, V δ 1 lymphocytes were significantly expanded (0.33% in healthy volunteers (HV) compared to 0.98% in the CLL group) (Fig. 1A). V δ 3–V δ 5 subset was gated among V δ 1- and V δ 2-negative cells (Fig. 1B). Next, we assessed the expression of NKp30 and CD226 on each subset, again without any significant differences (Fig. 2,3). Finally, we assessed the expression of checkpoint molecules on V δ 1 and V δ 3–V δ 5 subsets. No significant differences were noted, except for the downregulation of CTLA-4 on V δ 1 (2.99% in HV compared to 1.67% in CLL) (Fig. 4)

CD226 expression is higher in ZAP-70 negative cases

Next, we checked whether CD38 and ZAP-70 status significantly affected the immune parameters we tested. Out of all the markers, only CD226 MFI increased significantly between ZAP-70 positive and negative cases (19,041 compared to 15,058) (Fig. 5A). No differences were noted for the remaining parameters (TIGIT, PD-1, CTLA-4, NKp30) (data not shown).

Patients with known deletions have lower CD226 expression on V δ 2 cells

Out of the total group, 8 patients had some identified deletions, being mostly *TP53* deletions. Thus, we divided patients into those with and without known deletions. Then, we compared the immunological parameters between those 2 groups. Patients with identified deletions

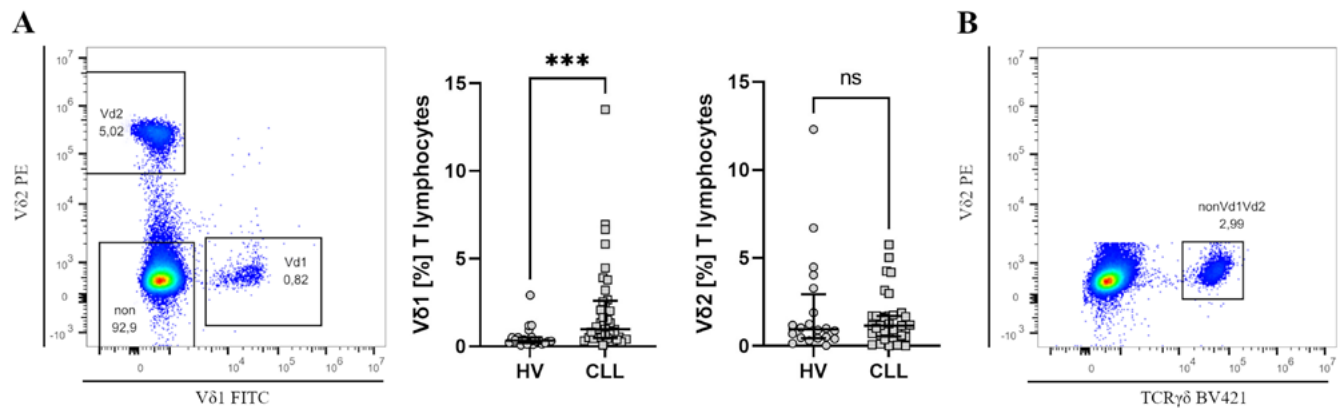


Fig. 1. The identification of $\gamma\delta$ T subsets along with CD226 and NKp30 expression. Vδ1 and Vδ2 cells were gated among CD3⁺ lymphocytes (A), while non-Vδ1–Vδ2 were gated from the negative population (non in A) and then gated as TCR $\gamma\delta$ ⁺ HV (healthy volunteers) (B)

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

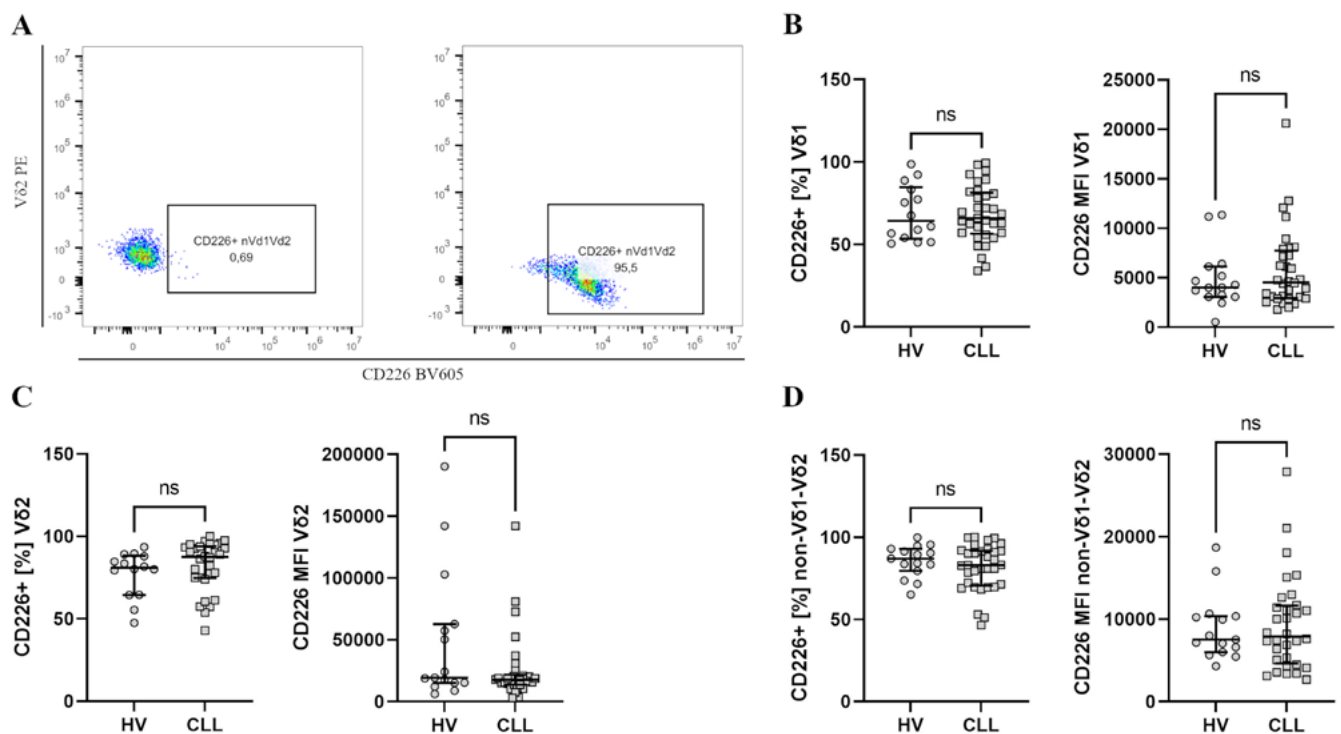


Fig. 2. CD226 expression on $\gamma\delta$ T cells. The gating for CD226 was controlled with FMO (fluorescence minus one) controls (A); results are presented separately for each subset – Vδ1 (B), Vδ2 (C) and non-Vδ1–Vδ2 (D)

HV – healthy volunteers; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

exhibited significantly lower expression of CD226 on Vδ2 cells compared to those without deletions (77.50% compared to 91.60%). (Fig. 5A).

The percentage of Vδ1 drops with the disease stage, but the expression of CD226 thereon increases

Rai classification is commonly used for the clinical staging of CLL. Thus, we divided patients into 3 groups: stage 0, stages I–II and stages III–IV. The percentage of Vδ1 trended down with an increase in the disease stage, although this

was not significant (Fig. 5B). Interestingly, we noted a significant increase in the expression of CD226 on Vδ1 cells (Fig. 5B). In contrast, CD226 expression on Vδ2 cells decreased with stage progression (Fig. 5B).

CD226/TIGIT ligands expression varies between IGVH mutated and unmutated subjects

Using a publicly available dataset, we assessed the expression of CD226/TIGIT ligands on neoplastic B cells in CLL. Only *PVR* (*CD155*) had a notable expression,

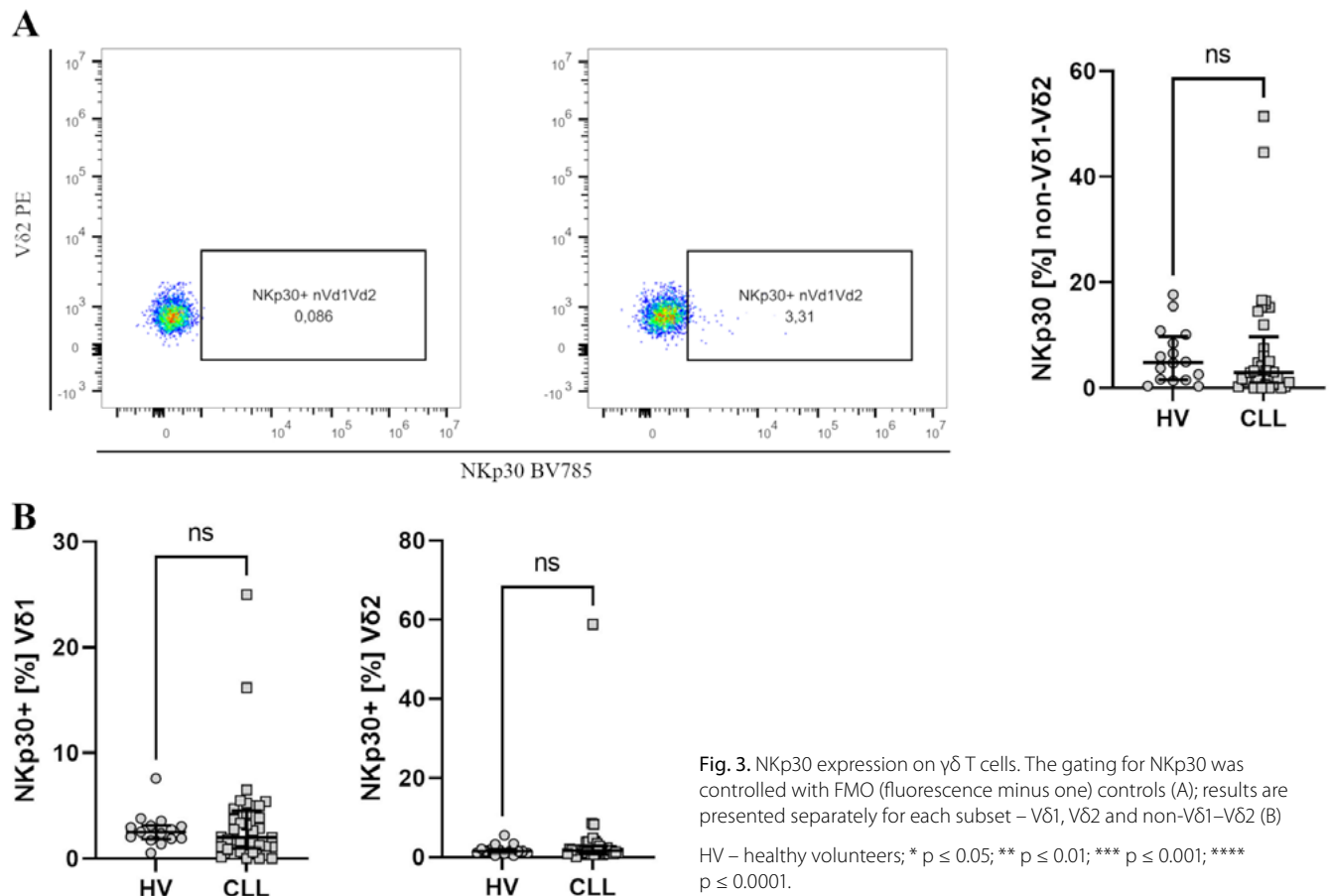


Fig. 3. NKp30 expression on $\gamma\delta$ T cells. The gating for NKp30 was controlled with FMO (fluorescence minus one) controls (A); results are presented separately for each subset – Vδ1, Vδ2 and non-Vδ1–Vδ2 (B)

HV – healthy volunteers; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

and both *NECTIN2* (*CD112*) and *NECTIN3* (*CD113*) had negligible expression levels (Fig. 6A). PVR expression was also significantly higher in IGVH unmutated subjects, and no differences were observed for the other 2 ligands (Fig. 6A). The overexpression of PVR was also confirmed with qPCR in isolated B cells from CLL patients and HV (Fig. 6B).

CD226 expression on Vδ2 correlates positively with IgA and IgG levels and negatively with LDH

A moderate positive correlation between CD226 ($\rho = 0.63$) expression on Vδ1 and Vδ3–Vδ5 was noted, and a similar correlation was observed for PD-1 ($\rho = 0.727$). CD226 expression on Vδ2 cells correlated moderately with lactate dehydrogenase (LDH) levels ($\rho = -0.637$), immunoglobulin A (IgA) ($\rho = 0.608$) and IgG ($\rho = 0.573$) serum concentrations. Moreover, IgG correlated with NKp30+ Vδ1 ($\rho = -0.529$), while similarly IgA correlated with TIGIT+ Vδ1 ($\rho = 0.6$) (Fig. 7). For the remaining correlations, the monotonic component of the relationship was not detected, and they are presented in the full matrix of correlations for immunological, hematological and clinical parameters (Supplementary Fig. 1,3).

Discussion

Chronic lymphocytic leukemia, especially a relatively advanced disease, is characterized by an accumulation of highly immunosuppressive cells, e.g., monocytic myeloid-derived suppressor cells (MDSCs) or B regulatory cells (Bregs).^{20,21} Such an immunosuppressive environment is believed to promote co-inhibitory checkpoint molecule expression.²² The expression of co-inhibitory and co-stimulatory molecules, e.g., PD-1, on Vδ2 was previously studied in CLL with some significant differences noted.^{23,24} Thus, we focused on non-Vδ2 cells, which are currently lacking investigations. Most importantly, we did not observe any significant upregulation of CTLA-4, PD-1 or TIGIT in either Vδ1 or Vδ3–Vδ5 subsets. This suggests that Vδ1 cells may be less affected by the highly immunosuppressive environment of peripheral blood from CLL patients. Indeed, previous observations, e.g., good response to autologous neoplastic B cells, high cytotoxic potential or good in vitro proliferation, seem to confirm this.^{9,14,15} This is in sharp contrast to Vδ2 cells, which tend to be exhausted and dysfunctional in CLL. Moreover, even Vδ2 cells obtained from healthy individuals provide significantly weaker responses to neoplastic B cells.^{13,23–25}

CD226, also known as DNAM-1, is an activating receptor important for cytotoxicity of NK and $\gamma\delta$ T cells. It competes

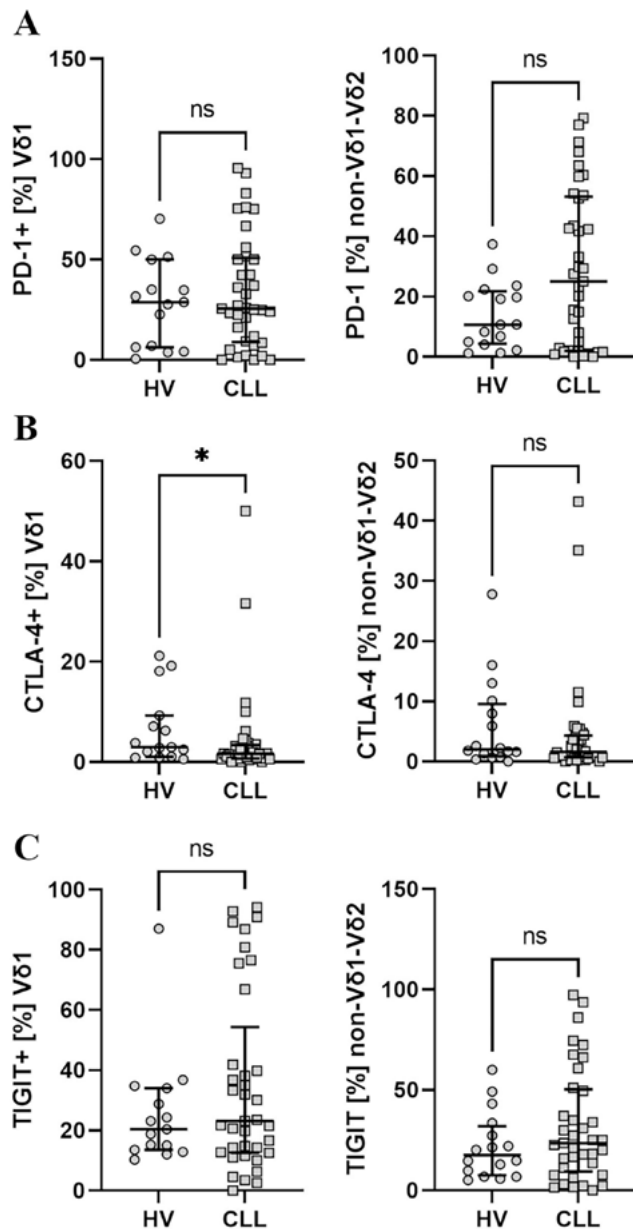


Fig. 4. Expression of checkpoint molecules: PD-1 (A), CTLA-4 (B) and TIGIT (C) was assessed using flow cytometry. Gating was set according to the FMO (fluorescence minus one) controls (presented in Supplementary Fig. 1)

HV – healthy volunteers; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

with TIGIT for its ligands, namely PVR (CD155), CD112 and CD113.^{26,27} Reduced expression of CD226 on $\gamma\delta$ T cells results in decreased cytotoxic potential in neuroblastoma.⁸ No differences in CD226 expression were noted for any of the subsets in the current study. This is similar to the observation of CD226 expression on NK cells in CLL.²⁸ While Veuillen et al. argued that CD226 ligands are rarely expressed by neoplastic B cells in CLL,²⁹ contrary results were obtained from RNAseq data analysis, namely, a significantly upregulated expression of *PVR* (*CD155*) was

noted. TIGIT is a novel checkpoint molecule that was discovered in 2009.³⁰ TIGIT competes with CD226 for their ligands, and studies with blocking antibodies against TIGIT demonstrated that it comprises mostly the Th1-related and cytotoxic response.³¹ In this study, we noted an insignificant increase in TIGIT expression on non-V δ 2 cells. This is partly comparable to the situation in acute myeloid leukemia (AML) where, on the one hand, TIGIT expression is increased and, on the other, CD226 is decreased.³²

Similar to Bartkowiak et al., we observed a significant expansion of V δ 1 in CLL patients,³³ whereas we noted only a small and statistically insignificant expansion of V δ 2 cells, which is in line with de Weerd et al., who also noted an insignificant accumulation of V δ 2.²³ Interestingly, despite the drop in V δ 1 percentage in patients with more advanced disease, we observed an increase in CD226 expression on V δ 1. This may suggest that V δ 1 retains at least some of its activity and, hopefully, some of its cytotoxic potential. Almeida et al. proposed an optimized protocol for clinical-grade expansion of V δ 1 cells from CLL patients for further use in in vitro immunotherapy.¹⁴ The results of the current study suggest that V δ 1 cells are less affected by the CLL burden than V δ 2 ones, and with such a feasible clinical-grade expansion, they show a real potential for CLL immunotherapy. V δ 1 cells exhibit significant cytotoxicity against leukemic cells and have been observed to persist in the circulation for longer periods than V δ 2 cells.³⁴ Moreover, V δ 1, both from healthy donors and from CLL patients, are significantly more cytotoxic against CLL cells.^{13,15}

ZAP-70 and CD38 are important prognostic factors in CLL.³⁵ The standard cutoff (as in our study) for positive/negative ZAP-70/CD38 is 20% of positive B cells.³⁵ After such division, we noted a significantly lower expression of CD226 on V δ 2 cells from ZAP-70-positive patients. Moreover, we have also noted a decreasing trend in CD226 expression on V δ 2 in patients with more advanced disease. This suggests that V δ 2 may be more affected by the immunosuppressive environment of CLL than V δ 1. Lactate dehydrogenase level is commonly considered to be a marker of disease burden with an important prognostic value; the higher the LDH level, the more advanced the disease.^{36,37} Similarly, both IgA and IgG have prognostic value, and their low serum level is usually associated with an advanced and progressive disease.^{38,39} Thus, correlations of LDH, IgA and IgG on one side and CD226 expression on V δ 2 on the other seem to be a reflection of similar changes related to tumor burden and progressing dysfunction of V δ 2 cells. The presence of PVR overexpression, a known CD226/TIGIT-ligand, on neoplastic B cells suggests that the CD226/TIGIT–PVR axis may be a significant target for therapeutic modulations. In addition, since V δ 1 cells appear to be much less susceptible to CLL-induced immunosuppression, V δ 1 immunotherapy could be used together with CD226/TIGIT PVR modulators.

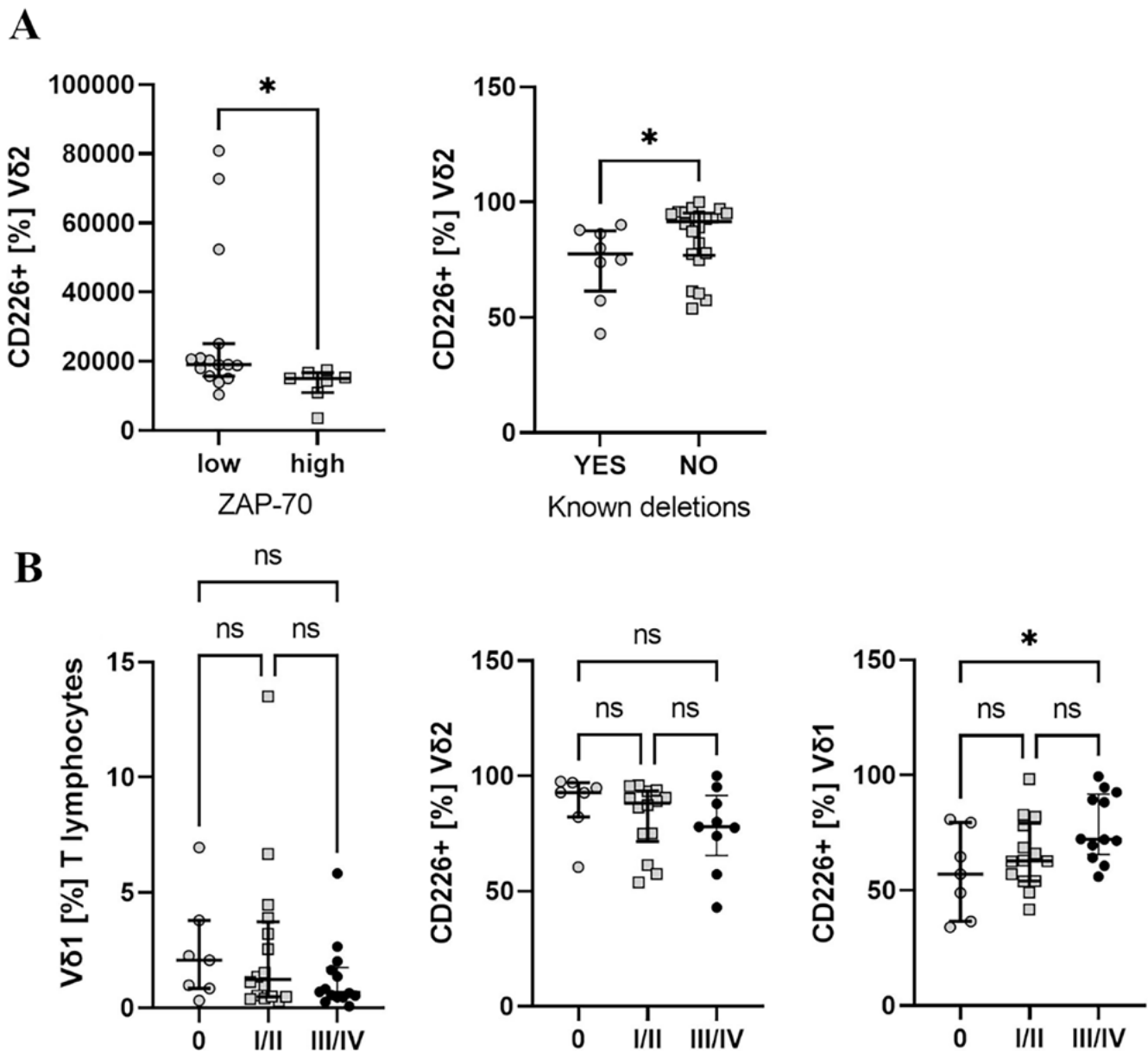


Fig. 5. Differences in immunological parameters in patients in high/low risk groups (cutoff point: 20% positive B cells). Each patient was screened for *TP53* and *ATM* deletions; no known deletions means that neither of those genes was affected (A); different stages of disease (B)

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

Limitations

The current study has some important limitations. First, the Vδ3–Vδ5 cells were analyzed as a whole due to the lack of appropriate monoclonal antibodies. Specifically, there are no anti-Vδ3, anti-Vδ4 or anti-Vδ5 antibodies available on the market. Moreover, the current study lacks any functional analyses. Due to COVID-19, we were experiencing a decrease in the number of patient samples and patients at more advanced stages of the disease reported to the clinic. Most samples did not allow for cell cultures and functional studies. Finally, as the inter-group comparisons and Spearman's correlation analysis were

pre-planned, we decided to employ no post hoc correction for p-value calculations which may have increased the risk of type I error.

Conclusions

Gamma-delta T cells are a heterogeneous group of T cells. The current study suggests that the non-Vδ2 subset of $\gamma\delta$ T cells is less affected by the immunosuppressive environment of CLL than the Vδ2 population. This is further corroborated by previously published results regarding cytotoxicity and activation triggered by CLL-derived B cells.

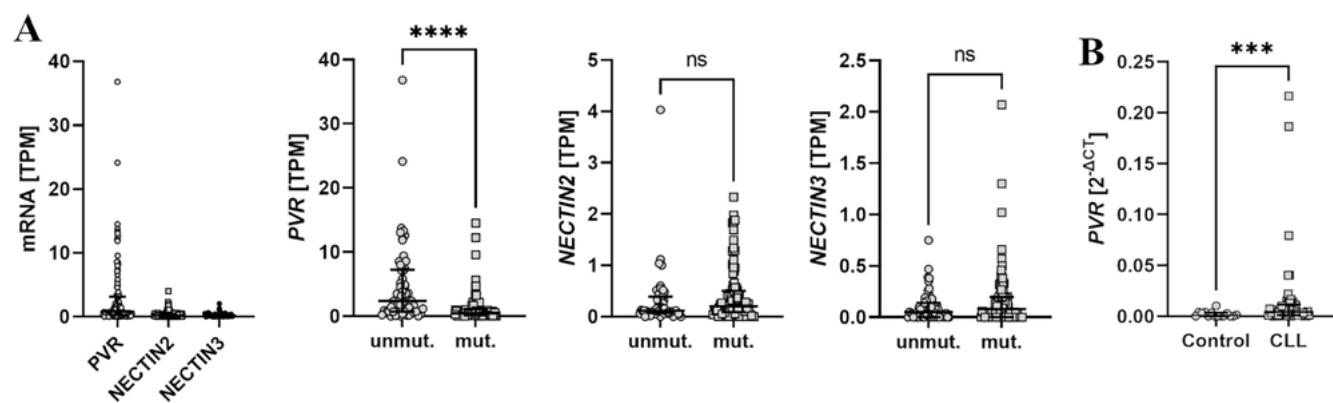


Fig. 6. Expression of CD226/TIGIT ligands on chronic lymphocytic leukemia (CLL) B cells was assessed in publicly available RNAseq datasets (A). PVR expression was assessed using real-time quantitative polymerase chain reaction (qPCR) in isolated B cells from CLL patients and healthy volunteers (B). Gene expression was normalized against *GAPDH*

unmut – unmutated IGVH; mut – mutated IGVH; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

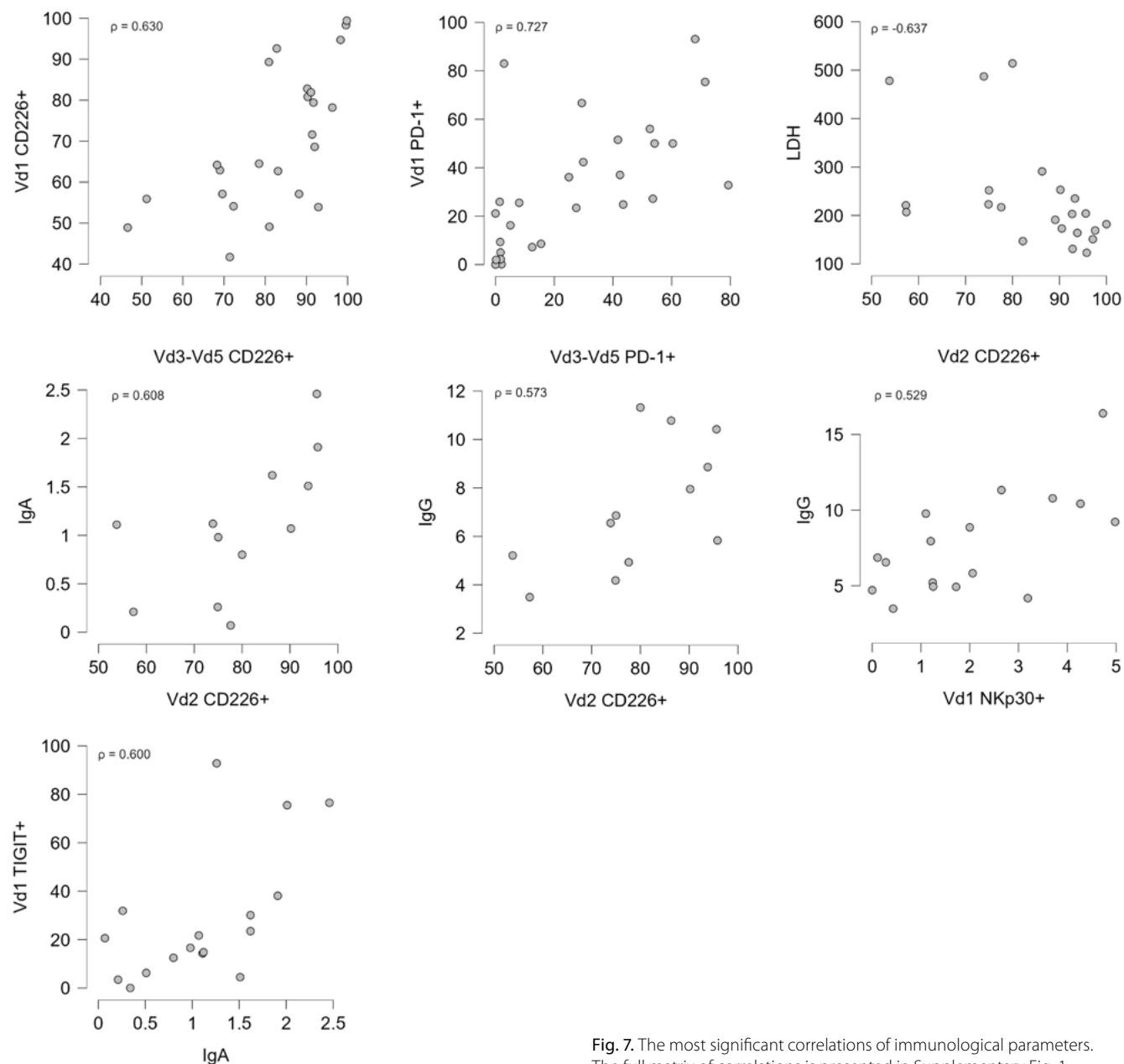


Fig. 7. The most significant correlations of immunological parameters. The full matrix of correlations is presented in Supplementary Fig. 1

Supplementary data

The Supplementary materials are available at <https://zenodo.org/doi/10.5281/zenodo.10848380>. The package includes the following files:

Supplementary Fig. 1. Gating strategy.

Supplementary Fig. 2. The full matrix of correlations.

Supplementary Fig. 3. Scatter plots for each correlation pair.

Supplementary Table 1. Exact p-values for intergroup comparisons.

Data availability


The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.


Consent for publication


Not applicable.


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