T$\gamma\delta$ LGL LEUKEMIA IDENTIFIES A SUBSET WITH MORE SYMPTOMATIC DISEASE: ANALYSIS OF AN INTERNATIONAL COHORT OF 137 PATIENTS

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Gregorio Barilà[1,2], Angela Grassi[3], HeeJin Cheon[4], Antonella Teramo[1,2], Giulia Calabretto[1,2], Jasmanet Chahal[4], Cristina Vicenzetto[1,2], Julia Almeida[5], Bryna C. Shemo[4], Min Shi[6], Vanessa Rebecca Gasparini[1,2], Noemi Munoz-Garcia[5], Cédric Pastoret[7], Hideyuki Nakazawa[8], Kazuo Oshimi[9], Lubomir Sokol[10], Fumihiro Ishida[8], Thierry Lamy[7], Alberto Orfao[5], William G. Morice[6], Thomas P. Loughran Jr[4], Gianpietro Semenzato[1,2] and Renato Zambello[1,2]

[1]Department of Medicine (DIMED), Hematology and Clinical Immunology section, Padova University School of Medicine, Padova, Italy
[2]Veneto Institute of Molecular Medicine (VIMM), Padova, Italy
[3]Immunology and Molecular Oncology Unit, Veneto Institute of Oncology, IOV-IRCCS, Padova, Italy
[4]Department of Medicine and University of Virginia Cancer Center, Division of Hematology & Oncology, University of Virginia School of Medicine, Charlottesville, USA
[5]Cancer Research Center (IBMCC-CSIC/USAL), Department of Medicine and Cytometry Service, University of Salamanca, IBSAL and CIBERONC, Salamanca, Spain
[6]Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, USA
[7]Department of Hematology, Rennes University Hospital, Rennes, France
[8]Department of Biomedical Laboratory Sciences, Shinshu University School of Medicine, Matsumoto, Japan
[9]Department of Medicine, division of Hematology, Kushiro Rosai Hospital, Japan
[10]Department of Malignant Hematology, Moffitt Cancer Center, Tampa, Florida, USA

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Corresponding authors:
Prof. Renato Zambello,
Department of Medicine (DIMED),
Padua University School of Medicine,
Via Giustiniani 2, 35128 Padova, Italy.
Phone: ++ 39 049 8218651
Fax: ++ 39 049 8211970
E-mail: r.zambello@unipd.it

Prof. Gianpietro Semenzato,
University of Padua,
Veneto Institute of Molecular Medicine,
Via Orus 2, 35129 Padova, Italy.
Phone: ++39 049-792-3217
Fax: ++ 39 049 792-3250
E-mail: g.semenzato@unipd.it

Keypoints

- *STAT3* mutations and Vδ2 *status* is needed to properly stratify Tγδ LGLL patients
- Independently from *STAT3* mutations, Tγδ LGLL represents a subset of T-LGLL characterized by dismal outcome as compared to Tαβ LGLL.

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ABSTRACT

Tγδ LGL Leukemia (Tγδ LGLL) is a rare variant of T-LGLL that has been less investigated as compared with the more frequent Tαβ LGLL, particularly in terms of frequency of *STAT3* and *STAT5b* mutations. In this study, we characterized the clinical and biological features of 137 patients affected by Tγδ LGLL retrospectively collected at 8 referral centers and collected from 1997 to 2020.

Neutropenia and anemia were the most relevant clinical features, being present in 54.2% and 49.6% of cases, respectively, including severe neutropenia and anemia in around 20% of cases each. Among the various treatments, Cyclosporine A was shown to provide the best response rates. DNA samples of 97 and 94 patients were available for *STAT3* and *STAT5b* mutations analysis, with 38.1% and 4.2% of cases being mutated, respectively. Clinical and biological features of our series of Tγδ patients were also compared with a recently published Tαβ cohort including 129 patients. Though no differences in *STAT3* and *STAT5b* mutational frequency were found, Tγδ cases more frequently presented with neutropenia (p=0.0161), anemia (p<0.0001), severe anemia (p=0.0065) and thrombocytopenia (p=0.0187). Moreover, Vδ2 negative patients displayed higher frequency of symptomatic disease. Overall, Tγδ patients displayed reduced survival with respect to Tαβ patients (p=0.0017).

Although there was no difference in *STAT3* mutation frequency, our results showed that Tγδ LGLL represents a subset of T-LGLL characterized by more frequent symptoms and reduced survival as compared to Tαβ LGLL.

INTRODUCTION

Large Granular Lymphocyte Leukemia (LGLL) is a rare and heterogenous chronic lymphoproliferative disorder characterized by the clonal expansion of Large Granular Lymphocytes (LGLs)[1,2]. The etiology of LGLL is unknown but a constitutive activation of JAK/STAT pathway is involved in the pathogenesis of LGL proliferation[3], further supported by the discovery of somatic *STAT3* and *STAT5b* mutations in approximately 40% of patients[4-9]. Among LGLLs, the latest WHO classification recognizes a CD3+ T-LGLL and CD3-NK-LGLL, accounting for 85% and 15% of cases, respectively. Moreover, based on surface T cell receptor expression, Tαβ and Tγδ subsets of LGLL can be identified[10].

While LGLL incidence ranges between 0.2 to 0.72 cases per 1 million individuals per year[1], the frequency of Tγδ proliferation is still not well defined, and most information has been collected through small retrospective studies. As compared to the more frequent Tαβ LGLL, Tγδ LGLL has been less investigated. Firstly reported by Oshimi et al. in 1988 in a 60 years old female exposed to the radiation in Nagasaki in 1945[11], Tγδ LGLL has been described in a sizable number of patients in 2006 by Sandberg et al. who reported an immunophenotypical analysis of 44 cases[12]. Up to now, only 4 retrospective studies including more than 200 LGLL patients are available[7,13-15], however few cases of Tγδ LGLL were included and only in the Italian cohort[7]. Consequently, the clinical features of Tγδ LGLL and information on the efficacy of treatments in this LGLL variant are still missing. Furthermore, data on the frequencies of *STAT3* and *STAT5b* mutations are nowadays available in Tαβ LGLL, but still limited and controversial in Tγδ LGLL. The Italian group recently reported 25% and 19% of Tγδ cases mutated in *STAT3* and *STAT5b* genes, respectively[7], while *STAT3* mutations were found in all patients included in a small Japanese Tγδ LGLL cohort[16].

With this as a background and lacking large cohorts of Tγδ patients, major referral groups dealing with LGLL were invited to join this collaborative study aimed at better characterizing Tγδ LGLL

patients, pointing to the evaluation of putative correlations among mutations, phenotype and clinical presentation, and the comparison of the clinical behavior of Tγδ LGLL with respect to the more common Tαβ variant. This large series of cases for the first time shows the dismal outcome of Tγδ LGLL with respect to Tαβ LGLL.

METHODS

Study patients

The study cohort included 137 patients affected by Tγδ LGLL followed from 1997 to 2020 at 8 referral centres across the world (France, Italy, Japan, Spain, USA). All patients met the currently approved WHO (World Health Organization) diagnostic criteria for T-LGLL[2,17]. T-LGL clonality was assessed by TCRγ gene rearrangement.

Demographic and clinical features, including presence of cytopenias, concomitant autoimmune/inflammatory diseases, secondary primary malignancies, treatment requirement and response, were collected. Response to treatment was evaluated based on periodical clinical and laboratory examinations after at least 4-6 months of therapy, using the currently accepted response criteria for LGLL[18]. The frequency of LGLs positive for the characteristic antigens was assessed by flow cytometry using direct immunofluorescence assays combining up to 6 markers/tube, according to standard operating procedures of individual centers. The investigation for LGL surface markers was performed on whole peripheral blood anti-coagulated with EDTA or ACD and on purified PBMCs. The commercially available FITC-, phycoerythrin (PE)-, PE-Cy5-, PE-Cy7-, APC- and APC-Cy7-conjugated mouse monoclonal antibodies (mAbs) used included: anti-CD3, anti-CD4, anti-CD8, anti-CD16, anti-CD56 and anti-CD57, anti-TCRγδ, anti-KIRs (killer Immunoglobulin-like receptors: CD158a, CD158b, CD158e), anti-NKG2A, anti-NKG2C, anti-Vγ9, anti-Vδ1 and anti-Vδ2 from Becton Dickinson (Sunnyvale, CA, USA).

This international Tγδ LGL leukemia cohort was compared with a recently reported equal size Italian Tαβ LGL leukemia cohort[7].

This study was performed according to the Helsinki Declaration and patients gave their written informed consent prior to inclusion in the study. The protocol and informed consent form were approved by the Padua ethics committee (approval number 4213/AO/17).

Screening for *STAT3* and *STAT5b* mutations

STAT3 and *STAT5b* sequencing was performed by Sanger Sequencing or Next Generation Sequencing according to local practice. For the screening of *STAT3* and *STAT5b* mutations by Sanger Sequencing, we used the set of primers reported by Koskela et al[4] and by Rajala et al[8], respectively, to amplify the hot spot regions for mutations (exon 19-21 for *STAT3* and exons 16-18 for *STAT5b*).

Statistical analysis

Patients' demographic, clinical and biological features expressed as categorical variables were compared by Fisher's exact test. Patient's overall survival (OS) was calculated from the date of diagnosis to death by any cause or the last known follow-up visit for censored patients. Survival curves were estimated using the Kaplan−Meier method and compared with respect to the patients' demographic and clinical characteristics with log-rank test. Schoenfeld residual testing was applied to assess the proportional hazards assumption. A univariate Cox proportional hazards regression analysis was employed to evaluate the prognostic relevance of each variable. Results for significant variables were presented as hazard-ratios (HR) and 95% confidence intervals.

To determine the effect of response to first-line treatment on PFS and OS we performed a 6-months landmark analysis in treated patients categorized by their response status (at least partial response vs. stable disease or progressive disease) at 6 months after the start of therapy. The 6-months landmark time was selected a priori, before the beginning of data analysis, since at least 4–6 months of treatment are recommended before correctly assess the response. For landmark analyses, PFS and OS were recalculated by shifting the time origin to 6 months after the start of therapy and patients who experienced the event of progression or death before this time were excluded from the PFS or OS landmark analyses, respectively.

A restricted mean survival time (RMST) analysis was also performed to compare the Tγδ and Tαβ LGLL cohorts. RMST is a robust and clinically interpretable summary measure of the survival time distribution, estimable even under heavy censoring and when the proportional hazards assumption is not satisfied, as an alternative to the hazard ratio approach[19,20]. This analysis depends on the truncation time point fixed for the RMST calculation. Four different truncation time points (100, 120, 140 and 160 months) were evaluated for the comparison of Tγδ and Tαβ LGLL cohorts. P values < 0.05 were considered significant. Statistical analysis was conducted using R version 3.6.2.

RESULTS

Clinical and Immunophenotypic features of Tγδ LGLL patients

Clinical and biological features of patients under study are summarized in Supplementary Table 1. Median age at diagnosis was 58.5 years (range, 18-92), with 29.4% of subjects being >65 years old. No relevant gender prevalence was clearly demonstrated (male 55.9%, female 44.1%). By immunophenotype, all patients showed an expansion of CD3+ TCRγδ+ T-cells, demonstrated to be clonal on molecular grounds. Tγδ LGLs usually displayed CD8 positivity (64/105, 61.0%), with 23 out 105 (21.9%) patients showing partial CD8 expression, otherwise CD4 was mostly absent, with only 3 cases showing partial expression. CD16 and CD57 were typical LGL markers, and they were expressed on the expanded Tγδ cells at the highest frequency (72.3% and 78.4%, respectively), while CD56 was present in 31.1% of cases. A dominant KIR expression was demonstrated in 23/56 patients (41.1%), with CD158b being the most frequently expressed marker (13/56, 23.2%), followed by CD158a (8/56, 14.3%) and CD158e (5/56, 8.9%). CD94/NKG2 receptor expression was

found in 32/75 cases (42.7%), 12 patients displaying NKG2A (12/54, 22.2%) and 3 patients showing NKG2C positivity (3/30, 10%).

Not being part of the work-up for the diagnosis of LGLL, bone marrow evaluation, either by flow cytometry or immunohistochemistry, was available for only 40/137 (29.2%) patients, showing variable degree of infiltration with a range from less than 1% to 60% of bone marrow cellularity.

Neutropenia -absolute neutrophil count (ANC) <1,500/mm^3- and mild anemia -hemoglobin (Hb) <120g/L- were the main relevant clinical features of the entire cohort, being present in 54.2% (65/120) and 49.6% (59/119) of patients, respectively. Severe neutropenia (ANC<500/mm^3) and severe anemia (Hb<90g/L) were observed in 25/120 patients (20.8%) and in 25/119 cases (21%), respectively. Thrombocytopenia (PLTs <100,000/mm^3) and splenomegaly were detected in 18/119 (15.1%) and in 31/122 (21.4%) patients, respectively. Forty-nine patients (41.5%) were affected by concurrent autoimmune/inflammatory (AA) diseases, mostly rheumatoid arthritis (16/49), autoimmune hemolytic anemia (5/49) and pure red cell aplasia (5/49). Finally, secondary primary malignancies (SPM) were detected in 17/84 patients (20.2%), either at the time of diagnosis or during the follow-up. Seven SPM were hematological (three marginal zone lymphoma, one chronic lymphocytic leukemia, one myelodysplastic syndrome, one plasma cell dyscrasia and one systemic mastocytosis) and ten non-hematological neoplasms, including three cases of thymoma, three cases of thyroid neoplasms, one lung cancer, one prostatic cancer, one cervical cancer and one skin cancer.

Treatment of Tγδ patients

Overall, more than half (53.7%) of patients required therapy during the natural history of the disease. All these patients were treated according to currently accepted indications[2,21]. In detail, 8/58 (13.8%) patients started therapy due to severe neutropenia, 4/58 (6.9%) due to symptomatic

neutropenia, 14/58 (24.1%) for transfusion dependent anemia and 13/58 (22.4%) for symptomatic anemia, 6/58 (10.4%) cases due to combined severe neutropenia and symptomatic anemia while the remnant 5/58 (8.6%) for symptomatic concomitant autoimmune diseases. In 8 cases (13.8%) the primary diagnosis was settled by hematology centers without experience in LGLL and subsequently the patients were moved to the referral centers. Consequently, a clear treatment indication was not available.

Considering first line treatment, most patients (34/57, 59.6%) received methotrexate (MTX), while 26.3% (15/57) were treated with cyclosporine A (CyA) and only 10.5% (6/57) received cyclophosphamide (CTX). The remaining two patients received cladibrine and splenectomy as first line treatment. Response rates and the absolute numbers of cases are reported in Supplementary Figure 1 and Table 1. Overall response (ORR) and complete response (CR) rates were lower in MTX treated patients (26.9% and 7.7%, respectively) compared to patients who received CyA and CTX (ORR: 53.9% and 40%, respectively, CR: 23.1% and 40%, respectively), although the latter therapies were used in lower numbers of cases, particularly CTX. Four patients treated with MTX discontinued the treatment due to toxicity.

Among patients requiring treatment (N=57), landmark analyses for progression free survival (PFS) and overall survival (OS) were performed according to response status at 6 months since therapy initiation, only in the subsets of patients for whom precise timing of response was available (N=20 for PFS and N=29 for OS). Irrespective from the type of first line treatment, responders (patients reaching at least partial response) after 6 months from the start of therapy were characterized by an increase in PFS with respect to non-responders (HR=6.16, 95% CI: 0.77-50.00; log-rank test p=0.05) (Figure 1, Panel A). Notably, although with a p-value not statistically significant, responders at 6 months showed also longer OS as compared to non-responders (log-rank test p=0.13) (Figure 1, Panel B). These results suggest a possible prognostic role of early response to

first-line therapy that should be further addressed in future prospective studies, by systematically collecting response times.

STAT3 and *STAT5b* mutation analysis

DNA samples of 97 and 94 patients were available for *STAT3* and *STAT5b* mutational analyses, respectively. *STAT3* mutations were detected in 37 patients (38.1%), with a prevalence of variants as follows: Y640F was detected in 16 cases (43.2%), D661Y in 9 cases (24.4%), D661V and S614R in 2 cases each (5.4%), and the H410R, Q448E, G618R, E638Q, K658F and N647I variants were found in one case each (2.7%). In the Italian cohort, two patients showed the A662_N663delinsH deletion/insertion and an in-frame insertion, G656_Y657ins, as previously reported[7]. In contrast, *STAT5b* mutations were found in only 4 patients (4.2%), from whom 3 patients carried the N642H variant and one had the Y665F mutation. Of note, *STAT3* and *STAT5b* mutations were mutually exclusive in Tγδ LGLL patients, never being detected concurrently in the same patient.

From the phenotypic point of view, *STAT3* mutated patients were characterized by lower frequency of expression of CD56 (3.8% vs 56.1%, p<0.0001), Vδ2 (0% vs 50%, p=0.0003) and Vγ9 (25% vs 57.1%, p=0.04). In addition, they showed a higher frequency of neutropenia (65.7% vs 40.8%, p=0.0288), severe neutropenia (31.4% vs 12.2%, p=0.0519), anemia (55.9% vs 34.7%, p=0.0726), autoimmune/autoinflammatory disorders (59.4% vs 31.5%, p=0.0139). They more frequently required therapy (67.9% vs 37.5%, p=0.0169) (Table 2). In contrast, no significant differences were found between *STAT3* mutated and wild-type Tγδ LGLL patients regarding the frequency of cases with LGL counts >2,000/mm^3 (25% vs 15.2%, p=0.3824), expression of KIRs (20% vs 50%, p=0.1413) and CD94 (38.9% vs 57.1%, p=0.2542), thrombocytopenia (17.6% vs 14.3%, p=0.7628), splenomegaly (22.9% vs 20.8%, p=1) and SPM (21.4% vs 22.7%, p=1) (Table 2).

Differently than *STAT3* mutated cases, *STAT5b* mutated patients were mostly asymptomatic, with only one patient experiencing mild neutropenia and splenomegaly.

Vδ pattern of expression analysis

Tγδ cells usually express 5 different Vδ receptor families (from Vδ1 to Vδ5), Vδ2 being generally expressed in blood circulating Tγδ cells, while the other subsets are typically enriched in epithelia, liver, and spleen[22]. In our cohort, flow cytometric Vδ analysis was available in 51 patients, 17 cases (33.3%) resulted Vδ2 positive while the remaining 34 (66.7%) were Vδ2 negative. Within this latter subset of patients, 16/34 (47.1%) were Vδ1 positive while 18 cases were neither Vδ1 nor Vδ2 positive (Table 3).

Vδ2 positive patients displayed a higher frequency of expression of CD56 (100% vs 9.1%, p<0.0001), KIR (64.3% vs 18.8%, p=0.0236), CD94 (76.5% vs 42.9%, p=0.0351) and NKG2A (71.4% vs 6.2%, p=0.0004), while no significant differences were found (vs Vδ2 negative cases) regarding CD16 and CD57 expression (100% vs 81.8%, p=0.1412 and 100% vs 81.8%, p=0.1412, respectively). Interestingly, all Vδ2 positive cases showed concomitant Vγ9 expression (100%), while only a small fraction of Vδ2 negative patients was also Vγ9 positive (18.2%, p<0.0001).

From the clinical point of view, Vδ2 positive cases displayed a more indolent LGLL. They rarely presented with symptomatic disease including neutropenia (5.9% vs 65.6%, p<0.0001), severe neutropenia (0% vs 31.2%, p=0.0094), anemia (0% vs 56.2%, p<0.0001), severe anemia (0% vs 34.4%, p=0.0090), splenomegaly (0% vs 26.7%, p=0.0371), and concurrent autoimmune/inflammatory disease (6.2% vs 48.4%, p=0.0039), in the absence of treatment requirement (0% vs 54.5%, p=0.0007). Interestingly, *STAT* mutations were mutually exclusive in Vδ2 negative and Vδ2 positive patients, all *STAT5b* mutated cases being Vδ2 positive (p=0.0327), whereas all *STAT3* mutated patients were Vδ2 negative (p=0.0003) (Table 3).

Clinical and biological features of Tγδ vs Tαβ LGLL.

To get further insight into the unique clinical and biological features of Tγδ LGLL, we compared our cohort of patients with a recently published Tαβ LGLL cohort of comparable size[7] (Table 4). No significant differences in gender and age were found between the two disease subtypes (p=0.3906 and p=0.2408, respectively), while Tαβ LGLL cases generally showed higher LGL counts than Tγδ LGLL cases (LGL count >2,000/mm^3 in 54.3% vs 22% patients, respectively; p<0.0001). By immunophenotype, Tγδ LGLL displayed a significantly higher frequency of expression of CD16 (72.3% vs 45.7%, p<0.0001), CD94 (42.7% vs 14%, p<0.0001), NKG2A (22.2% vs 10.1%, p=0.0355) and CD158a (14.3% vs 4.7%, p=0.0330) together with an increased KIR expression (41.1% vs 27.9%, p=0.0876), while they showed lower frequency of CD56 (31.1% vs 48.1%, p=0.0106) and CD57 expression (78.4% vs 94.6%, p=0.0003). Regarding *STAT* mutations, no significant differences were found between Tγδ and Tαβ LGLL patients in the frequency of *STAT3* (38.1% vs 37.9%, respectively; p=1) and *STAT5b* mutations (4.8% vs 12.5%, respectively; p=0.1130).

From the clinical point of view, Tγδ LGLL patients more frequently showed symptomatic disease in terms of neutropenia (54.2% vs 38.8%, p=0.0161), anemia (49.6% vs 11.6%, p<0.0001), severe anemia (21% vs 8.5%, p=0.0065), thrombocytopenia (15.1% vs 5.4%, p=0.0187), and concurrent autoimmune/inflammatory diseases (41.5% vs 21.7%, p=0.0009) (Table 4).

The markedly different observation times of Tγδ-LGLL and Tαβ-LGLL patients prevented to use a Fisher's exact test for these comparisons since this could lead to major bias due to lack of consideration of the time variable. Consequently, for secondary primary malignancies and need for treatment the data and the related p-value were not available.

Survival analysis

All demographic, clinical and biological features were evaluated for association with overall survival (OS) in Tγδ LGLL patients. After a median follow-up of 48 months, the median OS of our cohort was not reached. Splenomegaly was the only variable significantly associated with a shortened OS (log-rank test p=0.0012), with an HR=0.18 (95% CI: 0.06–0.59) (Figure 2, panel A), while other clinical and biological features of the disease had no significant impact on patient OS, including those previously found to be relevant for Tαβ LGLL patients[7], i.e. *STAT3* and *STAT5b* mutation status, or the presence of severe neutropenia or anemia (Supplementary Figure 2).

Direct comparison of patients' OS between Tγδ LGLL and the more common Tαβ LGLL is likely to prove a poorer overall outcome for Tγδ LGLL patients vs. Tαβ LGLL (log-rank test p=0.017) (Figure 2, panel B). This result must be interpreted with caution, since the two cohorts have different median follow-up times (Tγδ LGLL, 4 years vs. Tαβ LGLL, 9 years) and the proportional hazards assumption seems not to be fully satisfied due to the lack of events in the Tγδ cohort from 143 months onwards. Given the rarity of Tγδ LGLL, it was not possible to increase the cohort size, consequently we provided a supplementary analysis using a different measure of the effect that does not require the proportional hazards assumption, i.e. the restricted mean survival time (RMST). This analysis confirms a significant disadvantage in terms of survival of Tγδ LGLL patients with respect to Tαβ LGLL (Supplementary Table 2).

DISCUSSION

Here we report on the largest cohort of Tγδ LGL leukemia patients described so far in the literature with data collected between 1997 and 2020, as the result of a collaborative study involving 8 LGLL referral centers across the world. For the first time, we evaluated the clinical and biological features of this rare subset of T-LGLL on a large number of patients, screened for *STAT3* and *STAT5b* mutations. Overall, our results showed that Tγδ LGLL represents a variant with higher frequency of symptomatic disease and reduced survival as compared to the most common Tαβ LGLL subtype, despite a similar frequency of *STAT3* and to a less extent of *STAT5b* mutations.

In the past LGLL was considered a unique chronic and indolent disease, except for a few patients presenting with very aggressive disease[23]. In recent years, however, a better understanding of this disorder has been achieved pointing out the need for therapy in a significant fraction of LGLL patients[6,7,24]. Data provided in this study further encourage distinguishing Tγδ LGLL from Tαβ LGLL, since Tγδ LGLL patients showed unique clinical and biological features. Even though characterized by lower LGL counts, Tγδ LGLs more frequently express the CD16 and CD94 receptors, while the CD56 adhesion molecule and the CD57 immunosenescence-associated protein are less commonly expressed. Most importantly, Tγδ LGLL patients more frequently displayed symptomatic disease due to anemia (often transfusion dependent) potentially partially explained by an increased frequency of autoimmune hemolytic anemia and pure red cell aplasia[25], and concomitant autoimmune diseases. Altogether, this translates into a poorer outcome as compared to the more common Tαβ subtype of LGLL.

Overall these results are not consistent with previously reported data that did not show clear clinical differences between Tγδ LGLL and Tαβ LGLL[26]; however, the T-LGLL cohort reported by Bourgault-Rouxel et al. included only a small number of Tγδ patients (20 cases) compared to the almost 200 Tαβ reported cases, which limits the robustness of the conclusions raised[26]. A possible

limitation to be considered in the explanation of the worst outcome in Tγδ LGLL could be related to a high frequency of late-stage diseases due to the challenging diagnosis. As a matter of fact, in our series Tγδ patients showed lower LGLs count and CD57 expression as compared to the more common Tαβ patients. The high frequency of symptomatic patients herein reported within the Tγδ LGLL cohort may account for the reduced overall survival in this LGLL subtype. Aside this potential bias in survival analysis, our data point to the recommendation to include the Tγδ immunophenotype in the diagnostic work-up of unexplained cytopenia.

Despite the comparable size, the Tγδ and Tαβ LGLL cohorts we studied are characterized by different median follow-up (48 vs. 108 months, respectively), moreover the Tγδ LGLL cohort, due to its retrospective nature, suffers for the presence of several censored data. These findings lead to certain limitations in the interpretation of results. For this reason, an additional RMST analysis has been provided to mitigate these limitations, confirming a significant survival disadvantage for Tγδ LGLL patients with respect to Tαβ LGLL. In future perspective studies aimed at comparing the two cohorts it could be interesting to carefully plan the data collection in order to analyze variables that may depend on observation time, e.g. SPM or need for treatment, with a more appropriate time-to-event approach, thus minimizing any bias due to different follow-up lengths.

According to retrospective studies including few and heterogenous series of patients[27-29], treatment of LGLL still relies on immunosuppressive therapy, where MTX and CTX are used upfront, while CyA is generally reserved for relapsed/refractory patients[1,2,21]. To date, only one published prospective trial evaluating the efficacy of immunosuppressive therapy in LGLL is available[30], and one prospective and randomized trial comparing MTX and CTX as first line therapy in LGLL is currently ongoing (NCT01976182). However, all these studies do not report on the frequency of Tγδ LGLL analyzed and their specific response to therapy. Unexpectedly, MTX treatment led to unsatisfactory response rates in our series of Tγδ LGLL patients, with ORR being

observed in less than a third of patients, including CR in a very limited number of cases (7.7%). In contrast, first line therapy with CyA turned out to provide higher efficacy, with almost half patients responding of whom 23.1% reached complete response. An association between Tγδ LGLL and Pure Red Cell Aplasia (PRCA) has been widely described and it is also known that PRCA patients benefit from CyA treatment. In our cohort, treatment indication for the CyA cohort was available for 14 cases and 12 patients started therapy due to anemia, in 8 cases transfusion dependent; the remaining 2 cases had concomitant diagnosis of PRCA. It can be argued that PRCA has been underestimated in Tγδ LGLL with anemia/severe anemia, thus explaining the high overall and complete response rates obtained with CyA in this subgroup of patients. Of notice, the choice of the appropriate therapy is of utmost clinical relevance since we demonstrated here that responding patients were also characterized by a prolonged PFS and an improved overall survival. These data could offer a rationale for investigating CyA in the first line treatment of Tγδ LGLL, e.g. in new prospective trials.

Altogether, the above results indicate that, besides the distinction between T-LGLL and NK-LGLL, further dissection of T-LGLL into the Tαβ and Tγδ LGLL disease variants is of clinical relevance due to the poorer outcome and distinct treatment response profile of the latter patients. It is also worth noting that Tγδ LGLL cases did not appear as a homogeneous disease entity. Vδ2 positivity was associated with an immunophenotype characterized by Vγ9, CD56, KIR and CD94/NKG2A expression and, on clinical grounds, by lower frequency of symptomatic disease in terms of neutropenia, anemia, splenomegaly concomitant autoimmune/inflammatory disease and need of treatment compared to Vδ2 negative patients. Furthermore, the Vδ2 expression profile also correlated with the *STAT* mutational status since all *STAT3* mutated cases were Vδ2 negative, while the three *STAT5b* mutated patients were Vδ2 positive. Interestingly, the two subsets of Tγδ LGLL defined by the Vδ2 expression profile are likely to identify distinct cells of origin of Tγδ LGLL[22].

In line with this hypothesis, Vδ2 positive Tγδ LGLL might represent the neoplastic counterpart of blood circulating Tγδ cells, while Vδ2 negative Tγδ LGLL might mostly originate from tissue-derived Tγδ cells, with potential pathogenic implications.

Accumulating evidence indicates that the association between *STAT3* mutation and symptomatic disease is already recognized in Tαβ LGLL[6,7,31], recent data also supporting a reduced survival for *STAT3* mutated vs *STAT3* wild-type patients[7]. In contrast, the clinical impact of *STAT5b* mutations is still matter of debate, this mutation being present in the rare aggressive variants of LGLL[8] as well as in indolent CD4+ T-LGLL[7,32]. In the Tγδ LGLL setting, the real incidence of *STAT*s gene mutations is still unknown, being studied up to now only in small cohorts of patients[7,16,33]. In our study mutations in *STAT3* and *STAT5b* were screened in nearly hundred Tγδ LGLL patients and a frequency of *STAT3* mutations was found to be comparable with previously reported data in LGLL[4-6]. Moreover, we also detected 3 Tγδ LGLL patients harboring *STAT5b* mutations who displayed an indolent disease as observed in CD4+ Tαβ LGLL. In our cohort, we confirm the association between *STAT3* mutation and symptomatic disease, particularly with neutropenia, and increased need for therapy, although we did not observe a reduced overall survival for *STAT3* mutated cases. These results support a more aggressive disease behavior of Tγδ LGLL, and particularly for patients that do not show Vδ2 expression, independently from the *STAT3* mutational status.

In conclusion, data from this large multicentric cohort of Tγδ LGLL highlight the unique biological and clinical hallmarks of this rare variant of T-LGLL, likely associated with a discrete treatment response profile. Despite the similar frequency of *STAT3* and *STAT5b*, Tγδ LGLL cases in general, and Vδ2 negative Tγδ LGLL patients in particular, showed more symptomatic disease and a poorer outcome compared to Tαβ LGLL. At the same time, Tγδ LGLL patients appear to mostly benefit from cyclosporine as first line therapy. Altogether, these results underly the relevance of a precise characterization and subclassification of LGLL.

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Authorship Contributions

GB designed the research, analyzed data and wrote the manuscript. AG analyzed data, performed statistical analysis and wrote the manuscript. HJ, AT, GC, JC, CV, BS, VRG, NMG, HN and CP provided patient's samples and patient's data. JA, MS, KO, LS, FI, TL, AO, WGM and TL participated to the analysis of data and critically reviewed and edited the manuscript. GS provided funding, participated to the analysis of data, and critically reviewed and edited the manuscript. RZ designed the study, analyzed data, wrote the manuscript, and supervised the study.

Conflict of interest disclosure

The authors declare no competing financial interests.

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Table 1. Response to first line treatment.

		Response rate to first-line therapy			Treatment interruption
Treatment	N	ORR (%)	CR (%)	N/A	Toxicity
Methotrexate	34	7/26 (26.9%)	2/26 (7.7%)	4	4
Cyclosporine A	15	7/13 (53.9%)	3/13 (23.1%)	2	-
Cyclophosphamide	6	2/5 (40%)	2/5 (40%)	1	-
Cladribine	1	1/1 (100%)	0/1 (0%)	-	-
Splenectomy	1	0/1 (0%)	0/1 (0%)	-	-

Abbreviations. ORR: overall response rate. CR: complete response. N/A: not available.

Table 2: Biological and clinical features of *STAT3* mutated and *STAT3* wild-type Tγδ LGLL patients.

	***STAT3* mutated *n*=37**	***STAT3* wild-type *n*=60**	***p*-value**
Age>65 years	15/37 (40.5%)	11/59 (18.6%)	***p* = 0.0324**
LGL>2,000/mm^3	8/32 (25.0%)	7/46(15.2%)	*p* = 0.3824
CD16 expression	18/27(66.7%)	29/40(72.5%)	*p* = 0.7860
CD56 expression	1/26 (3.8%)	23/41 (56.1%)	***p* < 0.0001**
CD57 expression	21/27 (77.8%)	34/41 (82.9%)	*p* = 0.7541
KIR expression	2/10 (20.0%)	12/24 (50.0%)	*p* = 0.1413
CD158a expression	0/10 (0%)	3/24 (12.5%)	*p* = 0.5388
CD158b expression	1/10 (10.0%)	8/24 (33.3%)	*p* = 0. 2250
CD158e expression	1/10 (10.0%)	3/24 (12.5%)	*p* = 1
CD94 expression	7/18 (38.9%)	20/35 (57.1%)	*p* = 0.2542
NKG2A expression	1/10 (10.0%)	10/24 (41.7%)	*p* = 0.1133
NKG2C expression	1/8 (12.5%)	2/22(9.1%)	*p* = 1
Vδ1$^+$	7/12 (58.3%)	9/24(37.5%)	*p* = 0.2983
Vδ2$^+$	0/17 (0%)	17/34 (50.0%)	***p* = 0.0003**
Vγ9$^+$	4/16 (25.0%)	20/35(57.1%)	***p* = 0.0400**
ANC<1,500/mm^3	23/35 (65.7%)	20/49 (40.8%)	***p* = 0.0288**
ANC<500/mm^3	11/35 (31.4%)	6/49 (12.2%)	*p* = 0.0519
Hb<120 g/L	19/34 (55.9%)	17/49 (34.7%)	*p* = 0.0726
Hb<90 g/L	10/34 (29.4%)	6/49 (12.2%)	*p* = 0.0874
PLTs<100,000/mm^3	6/34 (17.6%)	7/49 (14.3%)	*p* = 0.7628
Splenomegaly	8/35 (22.9%)	11/53 (20.8%)	*p* = 1
Autoimmune/inflammatory diseases	19/32 (59.4%)	17/54 (31.5%)	***p* = 0.0139**
Secondary primary malignancies	6/28 (21.4%)	10/44 (22.7%)	*p* = 1
Need for treatment	19/28 (67.9%)	18/48 (37.5%)	***p* = 0.0169**

p-values are calculated using Fisher's exact test.
Abbreviations. KIR: Killer Immunoglobulin-like Receptor. ANC: Absolute Neutrophils' Count. Hb: Hemoglobin. PLTs: platelets.

Table 3: Biological and clinical features of Tγδ LGLL patients according to Vδ2 status.

	Vδ2$^+$ ***n*=17**	**Vδ2$^-$** ***n*=34**	***p*-value**
LGL>2,000/mm^3	2/17 (11.8%)	9/29(31.0%)	*p* = 0.1723
CD16 expression	14/14(100%)	18/22(81.8%)	*p* = 0.1412
CD56 expression	14/14 (100%)	2/22 (9.1%)	***p* < 0.0001**
CD57 expression	14/14 (100%)	18/22 (81.8%)	*p* = 0.1412
KIR expression	9/14 (64.3%)	3/16 (18.8%)	***p* = 0.0236**
CD158a expression	1/14 (7.1%)	1/16 (6.2%)	*p* = 1
CD158b expression	6/14(42.9%)	2/16(12.5%)	*p* = 0.1010
CD158e expression	3/14 (21.4%)	1/16 (6.2%)	*p* = 0.3155
CD94 expression	13/17 (76.5%)	12/28 (42.9%)	***p* = 0.0351**
NKG2A expression	10/14 (71.4%)	1/16 (6.2%)	***p* = 0.0004**
NKG2C expression	0/14 (0%)	3/16(18.8%)	*p* = 0.2276
Vγ9$^+$	17/17 (100%)	6/33(18.2%)	***p* < 0.0001**
***STAT3* mutated**	0/17 (0%)	17/34 (50%)	***p* = 0.0003**
***STAT5b* mutated**	3/17 (17.6%)	0/34 (0%)	***p* = 0.0327**
ANC<1,500/mm^3	1/17 (5.9%)	21/32 (65.6%)	***p* < 0.0001**
ANC<500/mm^3	0/17 (0%)	10/32 (31.2%)	***p* = 0.0094**
Hb<120 g/L	0/17 (0%)	18/32 (56.2%)	***p* < 0.0001**
Hb<90 g/L	0/17 (0%)	11/32 (34.4%)	***p* = 0.0090**
PLTs<100,000/mm^3	1/17 (5.9%)	4/32 (12.5%)	*p* = 0.6463
Splenomegaly	0/16 (0%)	8/30 (26.7%)	***p* = 0.0371**
Autoimmune/inflammatory diseases	1/16 (6.2%)	15/31 (48.4%)	***p* = 0.0039**
Secondary primary malignancies	4/14 (28.6%)	6/24 (25%)	*p* = 1
Need for treatment	0/14 (0%)	12/22 (54.5%)	***p* = 0.0007**

p-values are calculated using Fisher's exact test.

Abbreviations. KIR: Killer Immunoglobulin-like Receptor. ANC: Absolute Neutrophils' Count. Hb: Hemoglobin. PLTs: platelets.

Table 4: Biological and clinical features of the Tαβ and the Tγδ LGLL cohorts#.

	Tαβ LGLL* *n*=129	Tγδ LGLL *n*=137	*p*-value
Gender Male	65/129 (50.4%)	76/136 (55.9%)	*p* = 0.3906
Age>65 years	47/129 (36.4%)	40/136 (29.4%)	*p* =0.2408
LGL>2,000/mm^3	70/129 (54.3%)	24/109 (22.0%)	***p* < 0.0001**
CD16 expression	59/129 (45.7%)	73/101 (72.3%)	***p* < 0.0001**
CD56 expression	62/129 (48.1%)	32/103 (31.1%)	***p* = 0.0106**
CD57 expression	122/129 (94.6%)	80/102 (78.4%)	***p* = 0.0003**
KIR expression	36/129 (27.9%)	23/56 (41.1%)	*p* = 0.0876
CD158a expression	6/129 (4.7%)	8/56 (14.3%)	***p* = 0.0330**
CD158b expression	29/129 (22.5%)	13/56 (23.2%)	*p* = 1
CD158e expression	6/129 (4.7%)	5/56 (8.9%)	*p* = 0.3124
CD94 expression	18/129 (14.0%)	32/75 (42.7%)	***p* < 0.0001**
NKG2A expression	13/129 (10.1%)	12/54 (22.2%)	***p* = 0.0355**
NKG2C expression	5/129 (3.9%)	3/30 (10.0%)	*p* = 0.1740
***STAT3* mutated**	39/103 (37.9%)	37/97 (38.1%)	*p* = 1
***STAT5b* mutated**	12/96 (12.5%)	4/94 (4.8%)	*p* = 0.1130
ANC<1,500/mm^3	50/129 (38.8%)	65/120 (54.2%)	***p* = 0.0161**
ANC<500/mm^3	29/129 (22.5%)	25/120 (20.8%)	*p* = 0.7611
Hb<120 g/L	15/129 (11.6%)	59/119 (49.6%)	***p* < 0.0001**
Hb<90 g/L	11/129 (8.5%)	25/119 (21.0%)	***p* = 0.0065**
PLTs<100,000/mm^3	7/129 (5.4%)	18/119 (15.1%)	**p = 0.0187**
Splenomegaly	22/129 (17.5%)	31/122 (21.4%)	*p* = 0.1225
Autoimmune/inflammatory diseases	28/129 (21.7%)	49/118 (41.5%)	***p* = 0.0009**

p-values are calculated using Fisher's exact test.

** Tαβ LGLL cohort of comparable size[7]*

 # The markedly different observation times of Tγδ-LGLL and Tαβ-LGLL patients prevented to use a Fisher's exact test for these comparisons since this could lead to major bias due to lack of consideration of the time variable. Consequently, for secondary primary malignancies and need for treatment the data and the related p-value were not available.

Abbreviations. KIR: Killer Immunoglobulin-like Receptor. ANC: Absolute Neutrophils' Count. Hb: Hemoglobin. PLTs: platelets.

Figure Legends

Figure 1: Progression free survival and overall survival landmark analysis of Tγδ LGLL treated patients.
Kaplan-Meier curves showing 6-month landmark analysis for progression free survival (PFS) (Panel A) and overall survival (OS) (Panel B) of Tγδ LGLL patients achieving at least a partial response to first line therapy (Responders) compared to non-responding patients (Non-responders) at 6 months from the start of therapy. Curves were compared by log-rank test.
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Figure 2. Overall survival analysis of Tγδ LGLL patients.
Panel A: Overall survival analysis of the Tγδ LGLL cohort with respect to presence/absence of splenomegaly. Survival curves were estimated using the Kaplan–Meier method and compared by log-rank test.
Panel B: Overall survival comparison between Tαβ and Tγδ cohorts. With a median follow-up of 108 months (Tαβ) and of 48 months (Tγδ), median OS was not reached in both the cohorts. Survival curves were estimated using the Kaplan–Meier method and compared by log-rank test.

Figure 1: Progression free survival and overall survival landmark analysis of Tγδ LGLL treated patients.

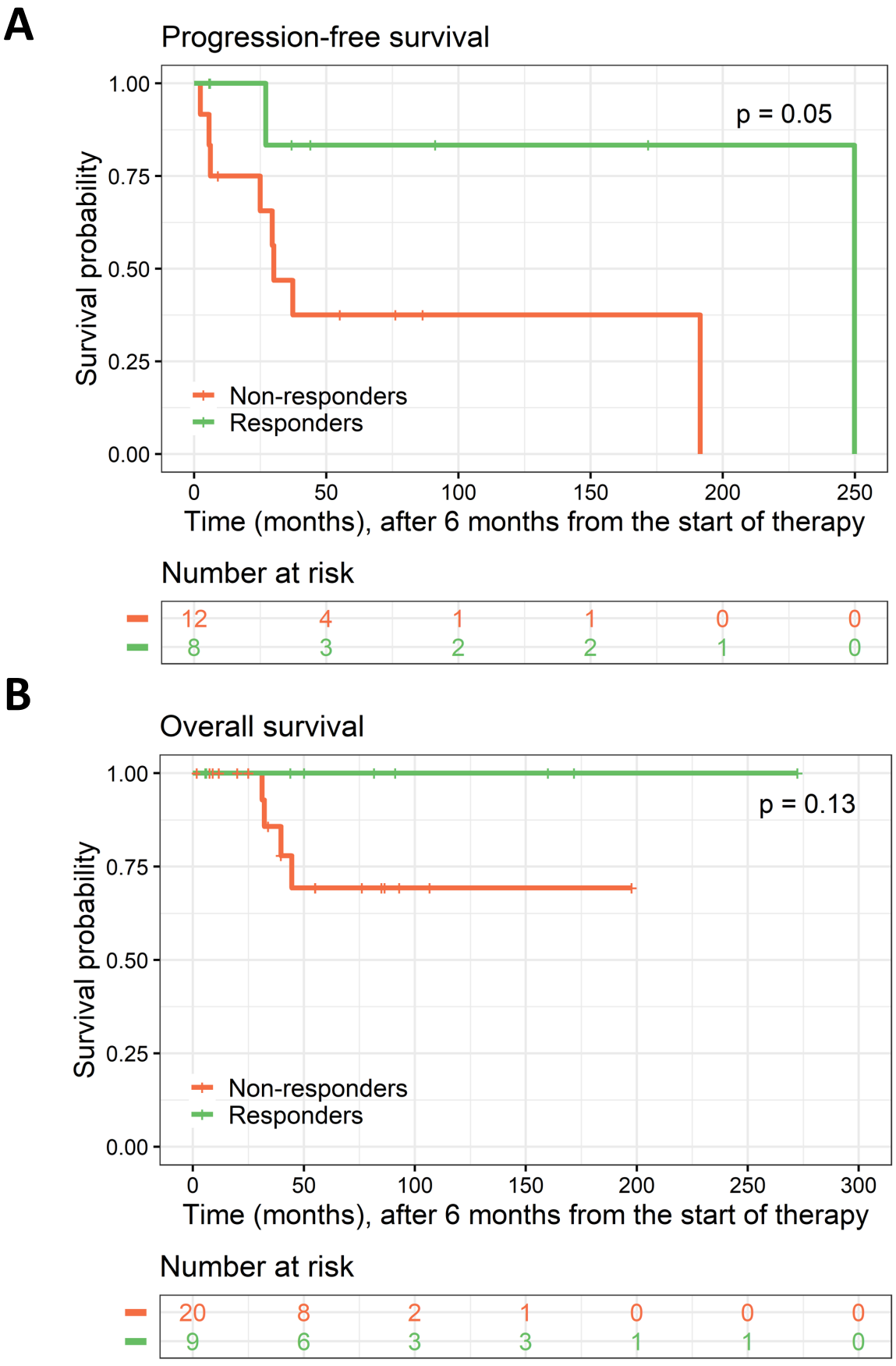

Figure 2. Overall survival analysis of Tγδ LGLL patients.