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Immunophenotypic heterogeneity of T-LGL Leukemia: clinical and biological implications

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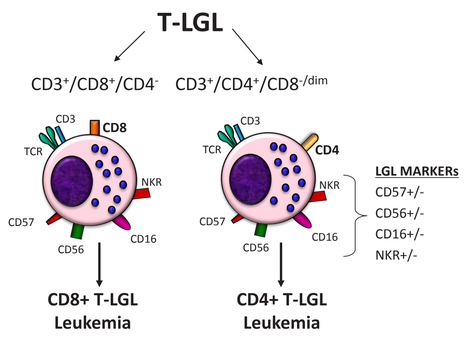


Figure 1. T-LGL schematic representation with its cell surface markers. $CD3^+/CD8^+/CD4^-$ T-LGL proliferation causes CD8+ T-LGLL while $CD3^+/CD4^+/CD8^{-/dim}$ causes CD4+ T-LGLL.

Background and Aim of the study

T-large granular lymphocytes leukemia is a rare disease characterized by an abnormal expansion of large granular lymphocytes (LGLs)[1,2]. T-LGLs typically exhibit a terminally differentiated cytotoxic T-cell phenotype (CD3+/CD8+/CD4–/αβTCR+)[3]. Anyway, together with the most common CD8+ T-LGLL, rare forms of CD4+/CD8-/+dim LGL proliferation (CD4+ T-LGLL) have been described[4]. In addition, LGLs can variably express CD57, CD16, CD56 and NK receptors (NKR), originating several possible immunophenotype combinations (Figure 1).

The disease generally has an indolent course, with neutropenia representing the major feature. About 85% of patients experience neutropenia during the natural history of the disease with near 40% presenting severe neutropenia[5].

Currently, the etiology of these diseases is still largely unknown. Several data reported that LGL proliferation is maintained for an impairment of the apoptotic machinery due to the activation of many survival signaling pathways. Among them a crucial role is played by JAK/STAT pathway, and in particular STAT3 is constitutively activated in leukemic LGLs resulting in oncogenes (i.e. Mcl-1[6,7]) induction. Recently, activating hot-spot STAT3 and STAT5b mutations have been discovered in T-LGLL patients supporting the idea that mutations could lead to a cytokine-independent STAT activation[8-10] (Figure 2). STAT3 mutations were described in 30-40% of T-LGLL patients[8,9], while STAT5b only in very few cases, these latters characterizing patients with aggressive clinical course[10].

The aim of this study was to correlate immunophenotypes with relevant biological and clinical features, namely STAT mutations and severe neutropenia in a series of 101 patients with T-LGLL.

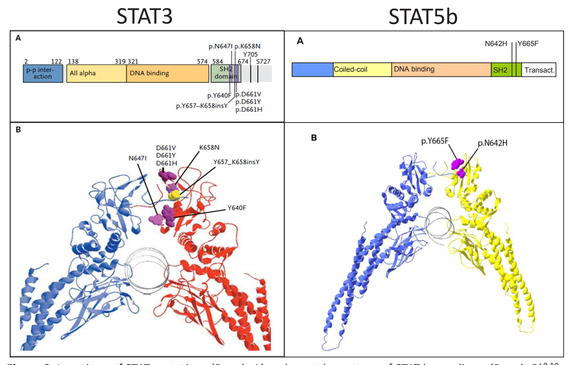


Figure 2. Locations of STAT mutations (Panels A) and crystal structure of STAT homodimer (Panels B)[8,10].

Patients and Methods

Patients and control donors. 101 patients were characterized by a chronic peripheral blood lymphocytosis (lasting more than 6 months) sustained by at least 2.000 LGLs/mm^3 (range: 25%-95% of lymphocyte pool). All patients met 2008 WHO criteria for T-LGLL diagnosis; clonality was demonstrated by molecular analysis of T-cell receptor (TCR) gene rearrangement. None of the patients had received treatment at the time of the study.

Isolation of LGLs. Mononuclear cells from peripheral blood (PBMC) were isolated through Ficoll (Sigma, St. Louis, MO) density centrifugation. By FACSAria cell sorter (BD biosciences, San Jose CA) or by micro beads and columns system (Miltenyi Biotec, Germany) purified T-type LGLs (CD57+ or CD16+) were obtained from PBMCs.

Flow cytometry analysis. The frequency of LGLs positive for the characteristic antigens was assessed by fluorescence-activated cell sorter (FACS) analysis using direct or indirect immunofluorescence assay combining 2 or 3 fluorescences. Cells were scored using a FACSCalibur analyzer (BD Biosciences) and data processed by the Macintosh CELLQuest software program (Becton Dickinson).

STAT mutations analysis. For the screening of STAT3 mutations (D661V, D661Y, D661H, Y640F, N647I, and K658N)[8] and STAT5b mutations (Y665F and N642H)[10] we used a set of primers[8,10] to amplify the exon 19, 20 and 21 of STAT3 and exon 16 of STAT5b, where all of the mutations are located. Sanger sequencing was performed on DNA from purified LGLs and from remaining autologous PBMCs. The presence of D661Y and Y640F STAT3 mutations undetectable by direct sequencing, because of the low sensitivity of the method (reaching 25% of positive cells) was also analyzed by a DNA tetraprimer amplification refractory mutation system assay (ARMS-PCR), as reported by Jerez et al[9].

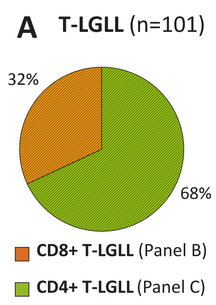
Statistical analysis. Data are expressed as mean ± the median standard error (SEM), and statistical analysis was performed by Student t test.
Comparisons of proportions and ranks of variables between groups were performed by the χ^2 test. A value of $p<0.05$ was accepted as significant.

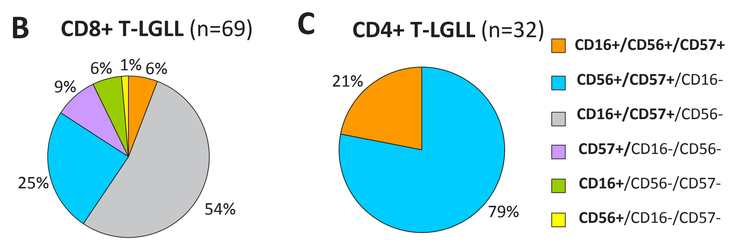
Results

1. Immunophenotypic characterization of patients T-LGL

By flow cytometer analysis, 69 of 101 (68%) patients resulted characterized by classical CD3+/CD8+/CD4- expression (CD8+ T-LGLL), while the remaining 32 patients belonged to the distinct CD4+ T-LGLL subtype with typical $CD3+/CD4+/CD8^{-/dim}$ staining (Panel A).

Afterward, we analyzed the variable expression of LGL markers, CD57, CD16 and CD56, making several immunophenotype combinations possible. In CD8+ T-LGLL patient group, six subgroups were recognized with the most frequent immunophenotype (37/69, 54%) characterized by CD16/CD57 positivity and CD56 negativity (Panel B). The CD4+ T-LGLL group exhibits only two phenotypes (Panel C).

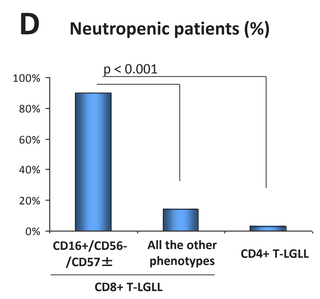


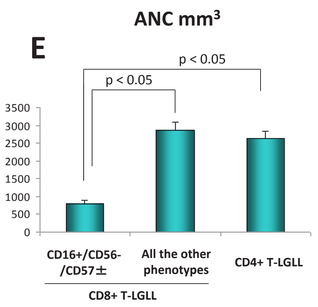


2. Evaluation of neutropenia in T-LGLL patients

Neutropenia (Absolute Neutrophil Count, ANC <1,500 mm3) was present in 39/101 patients (39%), with 17 presenting severe neutropenia (ANC<500 mm3) (Table 1). Neutropenia resulted statistically more frequent in females than males ($p<0.05$, Table 1).

All neutropenic patients were affected by CD8+ T-LGLL, except for only one CD4+ T-LGLL patient experiencing mild neutropenia (ANC=1,470 mm^3). In particular, 34 neutropenic patients were CD16+/CD57+/CD56- and 3 CD16+/CD57-/CD56-, suggesting that CD16+/CD56-/CD57± immunophenotype is associated with neutropenia manifestation (37/41, 90%, $X^2=74$, $p<0.001$). Only one neutropenic patient was CD57+/CD16-/CD56- and any neutropenic case was found in the other groups (Panel D). Consistently, the mean ANC in CD16+/CD56-/CD57± subgroup was significantly lower than that observed in both the other CD8+ and CD4+ T-LGLL patients (mean ANC±SEM: 803±591/mm^3, 2,861±1,190/mm^3; 2,636±1,105/mm^3, respectively, $p<0.05$; Panel E).

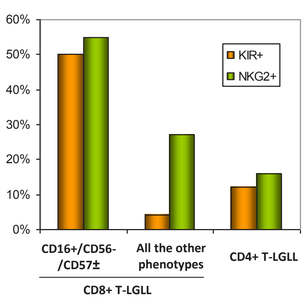




3. Characterization of NK receptor expression

KIR and NKG2 receptors expression was studied on leukemic LGLs by flow analysis. Twenty-seven out of 101 patients (27%) expressed at least one KIR and 36/101 (36%) expressed NKG2 receptors (Table 1).

In CD8+ T-LGLL, KIR and NKG2 receptor expression frequency was higher than in CD4+ T-LGLL (32% and 43% *vs* 12% and 15%, respectively, $p<0.005$). Among CD8+ T-LGLL, patients with CD16+/CD56-/CD57± phenotype had KIR and NKG2 expression frequency higher than the other CD8+ patients (50% and 55% vs 4% and 27%, respectively, $p<0.001$ for KIR expression). All the results are listed in Table 1 and represented in Panel F.



4. STAT3 mutations analysis

The analysis was performed on DNA purified from sorted LGLs of patients. By Sanger sequencing and ARMS-PCR we observed 37 patients (37%) carrying STAT3 mutations.

STAT3 mutations appeared to interest more frequently females than males ($p<0.05$, Table 2). All 37 STAT3 mutated patients belonged to CD8+ T-LGLL (n=69), in particular, 36 belong to CD16+/CD56-/CD57± subgroup (n=41) and only one showed CD57+/CD16-/CD56- phenotype. The association between CD16+/CD56-/CD57± immunophenotype and STAT3 mutations resulted statistically significant (Panel G, $p<0.001$).

A significant correlation was demonstrated between the presence of STAT3 mutations and neutropenia ($p< 0.001$, Panel H), and between the presence of STAT3 mutations and KIR and NKG2 expression ($p<0.001$, Table 2).

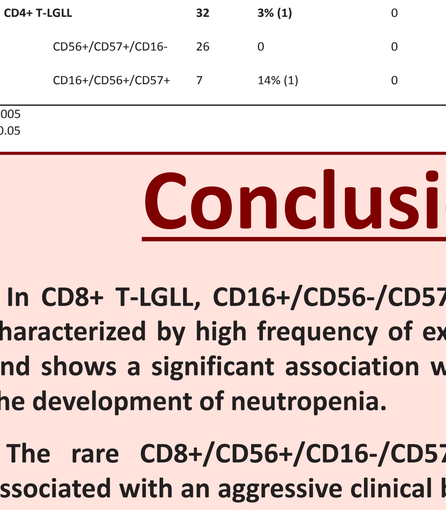


Table 1

	N. of pts	% of neutropenic pts (N)	% of severe neutropenic pts (N)	% of KIR+ pts (N)	% of NKG2+ pts (N)
T-LGLL	**101**	**39% (39)**	**17% (17)**	**27% (27)**	**36% (36)**
females	51	51% (26)**	25% (13)	25% (13)	47% (24)**
males	50	26% (13)	8% (4)	28% (14)	24% (12)
CD8+ T-LGLL	**69**	**55% (38)***	**25% (17)***	**33% (23)***	**45% (31)***
CD16+/CD57+/CD56-	37	92% (34)	38% (14)	54% (20)	51% (19)
CD16+/CD56-/CD57-	4	75% (3)	75% (3)	25% (1)	75% (3)
CD57+/CD16-/CD56-	6	17% (1)	0	0	0
CD56+/CD57+/CD16-	17	0	0	6% (1)	29% (5)
CD16+/CD56+/CD57+	4	0	0	0	75% (3)
CD56+/CD16-/CD57-	1	0	0	100% (1)	100% (1)
CD4+ T-LGLL	**32**	**3% (1)**	0	**12% (4)**	**16% (5)**
CD56+/CD57+/CD16-	26	0	0	8% (2)	12% (3)
CD16+/CD56+/CD57+	7	14% (1)	0	28% (2)	29% (2)

* p<0.005
** p<0.05

Table 2

Table 2. STAT3 mutation analysis in patients distinguished for LGL immunophenotype.

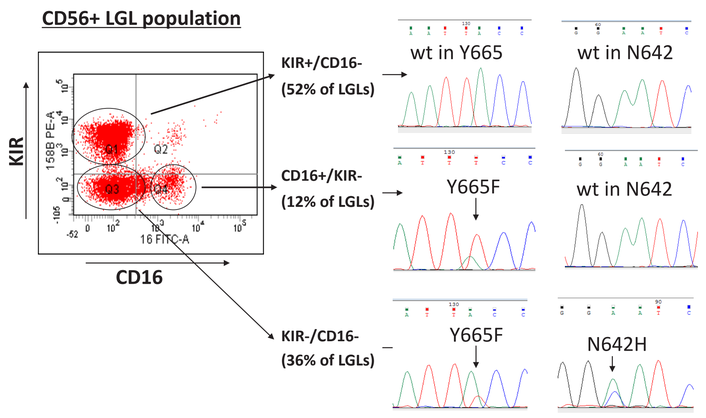
Variable	T-LGLL patients n=101	Patients with STAT3 mutation n=37	STAT3 mutation
Gender			
males	50	13 (26%)	8 Y640F, 2 D661Y, 1 D661V, 1 N647I, 1 K658R and I659_M660insL
females	51	24 (47%)*	16 Y640F, 6 D661Y, 1 D566N, 1 A662_N663delinsH
CD8+ T-LGLL	**69**	**37 (54%)***	**24 Y640F, 8 D661Y, 1 D661V, 1 N647I, 1 D566N, 1 K658R and I659_M660insL, 1 A662_N663delinsH**
CD16+/CD57+/CD56-	37	32 (86%)*	19 Y640F, 8 D661Y, 1 D661V, 1 N647I, 1 D566N, 1 K658R and I659_M660insL, 1 A662_N663delinsH
CD16+/CD56-/CD57-	4	4 (100%)*	4 Y640F
CD57+/CD16-/CD56-	6	1 (17%)	1 Y640F
CD56+/CD57+/CD16-	17	0	
CD16+/CD56+/CD57+	4	0	
CD56+/CD16-/CD57-	1	0	
CD4+ T-LGLL	**32**	**0**	
CD56+/CD57+/CD16-	26	0	
CD16+/CD56+/CD57+	7	0	
Neutropenia	39	33 (85%)*	21 Y640F, 7 D661Y, 1 D661V, 1 N647I, 1 D566N, 1 K658R and I659_M660insL, 1 A662_N663delinsH
Severe Neutropenia	17	17 (100%)*	10 Y640F, 5 D661Y, 1 N647I, 1 K658R and I659_M660insL
KIR+	27	18 (67%)*	10 Y640F, 5 D661Y, 1 N647I, 1 D566N, 1 A662_N663delinsH
NKG2+	36	20 (56%)*	14 Y640F, 3 D661Y, 1 N647I, 1 D566N, 1 A662_N663delinsH

* the association with STAT3 mutation results statistically significant ($p<0.005$)

5. STAT5b mutations analysis

In literature it is reported that CD8+/CD56+/CD16-/CD57- LGLL are associated with an aggressive clinical course. Recently, STAT5b mutations were reported to be associated with these rare cases.

In our patients' study group, the only case characterized by CD56+/CD16-/CD57- phenotype and a very aggressive disease, presented STAT5b mutations in his clonal LGLs (wild type for STAT3). Interestingly, this patient showed two different STAT5b mutations, N642H and Y665F, indicating the presence of subclones. By selecting for CD16 and KIR (CD158b) antigens expression in LGLs of this patient, three LGL subsets were recognized within the main CD3+/CD8+/CD56+ clone, namely a) KIR+/CD16-, b) KIR-/CD16- and c) CD16+/KIR- subsets. Performing mutation analysis on each of these cell subsets opportunely sorted, KIR+/CD16- cell subset resulted STAT5b wild type, KIR-/CD16- subset showed both N642H and Y665F mutations and the small CD16+/KIR- subpopulation carried Y665F mutation, largely getting over wild type nucleotide.



Conclusions

- **In CD8+ T-LGLL, CD16+/CD56-/CD57± immunophenotype is characterized by high frequency of expression of NK receptors and shows a significant association with STAT3 mutations and the development of neutropenia.**
- **The rare CD8+/CD56+/CD16-/CD57- immunophenotype is associated with an aggressive clinical behavior and with STAT5b mutations. Interestingly, different subclones characterized by different phenotypes and STAT5b mutations can be identified.**
- **CD4+ T-LGLL can be indicative of an indolent disease.**

➔ **LGL immunophenotype might be predictive of different biological and clinical features of disease**

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