Pro-inflammatory cells sustain leukemic clonal expansion in T-cell large granular lymphocyte leukemia

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Abstract

T-cell large granular lymphocyte leukemia (T-LGLL) is a chronic lymphoproliferative disorder characterized by the clonal expansion of T-cell large granular lymphocytes (T-LGL). Immunophenotypic and genotypic features contribute to discriminate symptomatic (CD8$^+$ *STAT3*-mutated T-LGLL) from clinically indolent patients, this latter group including CD8$^+$ wildtype (wt), CD4$^+$ *STAT5B*-mutated and wt cases. T-LGL lymphoproliferation is sustained both by somatic gain-of-function mutations (i.e., *STAT3* and *STAT5B*) and by pro-inflammatory cytokines, but little information is available on the activity of T-LGLL non-leukemic cells. In this study, we characterized pro-inflammatory cells in the peripheral blood of T-LGLL patients and analyzed their role in supporting the leukemic growth. In symptomatic patients we found that cell populations not belonging to the leukemic component showed a discrete pro-inflammatory pattern. In particular, CD8$^+$ *STAT3*-mutated cases showed a skewed Th17/Treg ratio and an abnormal distribution of monocyte populations characterized by increased intermediate and non-classical monocytes. We also demonstrated that monocytes released high levels of interleukin-6 after CCL5 stimulation, a chemokine specifically expressed only by leukemic LGL. Conversely, in asymptomatic cases an altered distribution of monocyte populations was not detected. Moreover, T-LGLL patients' monocytes showed abnormal activation of signaling pathways, further supporting the different pathogenic role of monocytes in patients in discrete clinical settings. Altogether, our data contribute to deepening the knowledge on the different cell subtypes in T-LGLL, focusing particularly on non-leukemic cell populations and thus offering the rationale for new therapeutic strategies.

Introduction

According to the 2022 World Health Organization (WHO) classification, T-cell large granular lymphocyte leukemia (T-LGLL) is the most frequent disorder (up to 85%) among chronic lymphoproliferative diseases of large granular lymphocytes (LGL). Affected patients show remarkable phenotypic and genotypic heterogeneity as well as a variety of clinical features, ranging from indolent to symptomatic patients requiring therapy and showing a dismal outcome.[1]
Recently, phenotypic and molecular analyses allowed a better categorization of T-LGLL patients. In particular, most symptomatic cases are included in CD8$^+$ T-LGLL and characterized by a CD3$^+$/CD8$^+$/CD16$^+$/CD56$^-$ clone, whereas the remaining patients presenting with either CD8$^+$ or CD4$^+$ T-LGLL, the latter characterized by CD4$^+$ or CD4$^+$/CD8^{dim} clones, are usually asymptomatic.[2,3] Many genes related to epigenetic modification have been found to be mutated frequently in T-LGLL patients, whose mutational status contributes to patients' stratification.[3,4] In particular, signal transducer and activator of transcription 3 (*STAT3*) mutations correlate with symptomatic disease in nearly 60% of CD8$^+$ T-LGLL cases,[5] while *STAT5B* mutations are found in 66%[6] of the asymptomatic CD4$^+$ T-LGLL group and in 100% of the rare aggressive variant of T-LGLL.[5-7] Symptomatic patients (38%) are characterized by cytopenias, with neutropenia and related infections being the most frequent clinical features.[5] Severe neutropenia (absolute neutrophil count <500/mm^3) and/or recurrent infections require treatment which, to date, includes immunosuppressive agents (methotrexate, cyclophosphamide and cyclosporine A).[8] T-LGLL may occur in association with auto-

immune diseases and, in particular, rheumatoid arthritis is diagnosed in roughly 20% of T-LGLL patients.[9] Interestingly, Felty syndrome, a rare variant of rheumatoid arthritis associated with splenomegaly and neutropenia, has been proposed as a diagnostic continuum due to the biological features consistent with T-LGLL, i.e., the identification of *STAT3* mutations and a similar pro-inflammatory cytokine pattern in the peripheral blood.[10] Moreover, the concurrence with autoimmune diseases is more frequently described in T-LGLL patients characterized by *STAT3* mutations.[3]

STAT3 is constitutively active in T-LGLL patients, also in cases devoid of the specific mutations,[2,11] this finding being considered as an essential factor for the survival of the leukemic clone.[12] The state of activation of T-LGL clones is sustained either by the gain-of-function mutations frequently found in the Src homologous 2 (SH2) domain of the gene, with Y640F and D661Y being the most recurrent, or by cytokine stimulation.[11,13] In particular, high levels of plasma interleukin (IL)-6 have been reported to elicit STAT3 phosphorylation and, in turn, to contribute to leukemic clone survival. In this setting, monocytes have been regarded as the main cellular source of IL-6.[13,14] Many other pro-inflammatory cytokines have been associated with the pathogenesis of T-LGLL, including Fas-L, which is linked to the development of neutropenia,[15] and CCL5, whose pathogenic mechanism has not been defined yet.[2,16,17] In addition, CCR5, one of the CCL5 receptors, has been reported to be less expressed on the surface of leukemic cells, particularly in neutropenic patients.[2,18]

Among the different players of the microenvironment, including cellular components (stromal, accessory and regulatory cells, among others) as well as soluble factors (cytokines and chemokines), in the present study we evaluated the role of the peripheral blood pro-inflammatory environment in the pathogenesis of T-LGLL also in relationship to different T-LGLL subgroups. In particular, we analyzed the non-leukemic cell subsets, namely Th17, Treg and monocytes, focusing on their involvement in sustaining the LGL clonal expansion. It is worth mentioning that these cell populations are also abnormally represented in patients with autoimmune diseases.[19,20] We herein provide information on the cytokine interplay demonstrating a relationship between monocytes and the leukemic clone in T-LGLL, particularly in symptomatic *STAT3*-mutated T-LGLL.

Methods

Patients

Fifty-eight patients and 27 healthy controls (HC, with matched age and sex) were enrolled in the study. The Padova Institutional Review Board approved this study and informed consent was collected from each subject in accordance with the Declaration of Helsinki. All patients met the 2022 WHO criteria for the diagnosis of T-LGLL.[1] No patients had received treatment at the time of the study. Their detailed clinical and diagnostic features are reported in *Online Supplementary Tables S1* and *S2*. Recruited patients were divided into four subgroups according to T-LGLL immunophenotype and the *STAT3* or *STAT5B* mutational status as previously described,[21] namely $CD8^+$ wildtype (wt) or *STAT3*-mutated and $CD4^+$ wt or *STAT5B*-mutated patients.

Cell isolation, purification and culture

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Hypaque (Sigma-Aldrich) gradient centrifugation. T-LGL and/or monocytes were immunomagnetically purified using magnetic microbeads coated with monoclonal antibodies against CD57 or CD56 for T-LGL or CD14 for monocytes (Miltenyi Biotec). The purity of the populations was checked by flow cytometry (Becton Dickinson, BD) and resulted >97%.

In selected cases, *in vitro* cultures were established. Cells derived from T-LGLL patients and HC were cultured at 2×10^6 cells/mL in complete RPMI-1640 (EuroClone) supplemented with 10% fetal calf serum (Sigma-Aldrich), 2 mM glutamine, 25 mM Hepes, 100 U/mL penicillin and 100 □g/mL streptomycin (EuroClone) and grown in 5% CO_2 at 37°C. PBMC and PBMC deprived of monocytes, derived from the same patient, were cultured for 1, 2, 3 and 5 days for the evaluation of activation of T-LGL apoptosis. For analysis of apoptosis and expression evaluations, purified monocytes and PBMC deprived of both T-LGL and monocytes were stimulated with 100 ng/mL CCL5 (R&D) for 12 h. Purified LGL were stimulated with 100 ng/mL IL-17A (CELLgs) for 24 h.

Flow cytometric analyses

The percentage of Th17 and Treg cells was assessed from PBMC using the commercial protocols Fix&Perm (ThermoFisher Scientific) and Foxp3 Buffer Set (BD), respectively. Th17 cells were stained with anti-human CD161 (FITC), IL-17 (PE), IL-23R (APC) and CD4 (APC-Cy7) and Treg with anti-human Foxp3 (PerCP-Cy5.5), CD25 (PE-Cy7) and CD4 (APC-Cy7, BD). For these analyses 500,000 events were acquired and the populations were reported as the percentage of $CD4^+$ cells.

The distribution of peripheral blood monocyte populations (namely classical [$CD14^{high}/CD16^{neg}$], intermediate [$CD14^{high}/CD16^{dim}$] and non-classical [$CD14^{dim/neg}/CD16^{high}$]) was determined by staining whole blood with anti-human CD66b (FITC), CD14 (PE), CD16 (PerCP-Cy5.5), CD19 (APC) and CD3 (APC-Cy7, BD).

IL-6 intracellular staining was performed following the commercial protocol Fix&Perm on whole blood treated for 6 h at 37°C with the exocytosis inhibitor Golgi Stop (BD)

and 100 ng/mL lipopolysaccharide (Sigma-Aldrich). For this analysis anti-CD14 (APC), CD16 (PerCP-Cy5.5) and IL-6 (PE, BD) were used.

Apoptosis was evaluated by staining LGL with anti-human CD57 (FITC) and CD8 (PerCP-Cy5.5) and the apoptotic cells with PE-conjugated annexin V (BD).

All the cytometric evaluations were performed with a BD FACS Canto II and the data processed by Diva Software (BD). The gating strategies are reported in *Online Supplementary Figures S1, S2* and *S3*.

Expression analysis and statistical evaluation

Details of the expression analysis and statistical evaluation are provided in the *Online Supplementary Methods*.

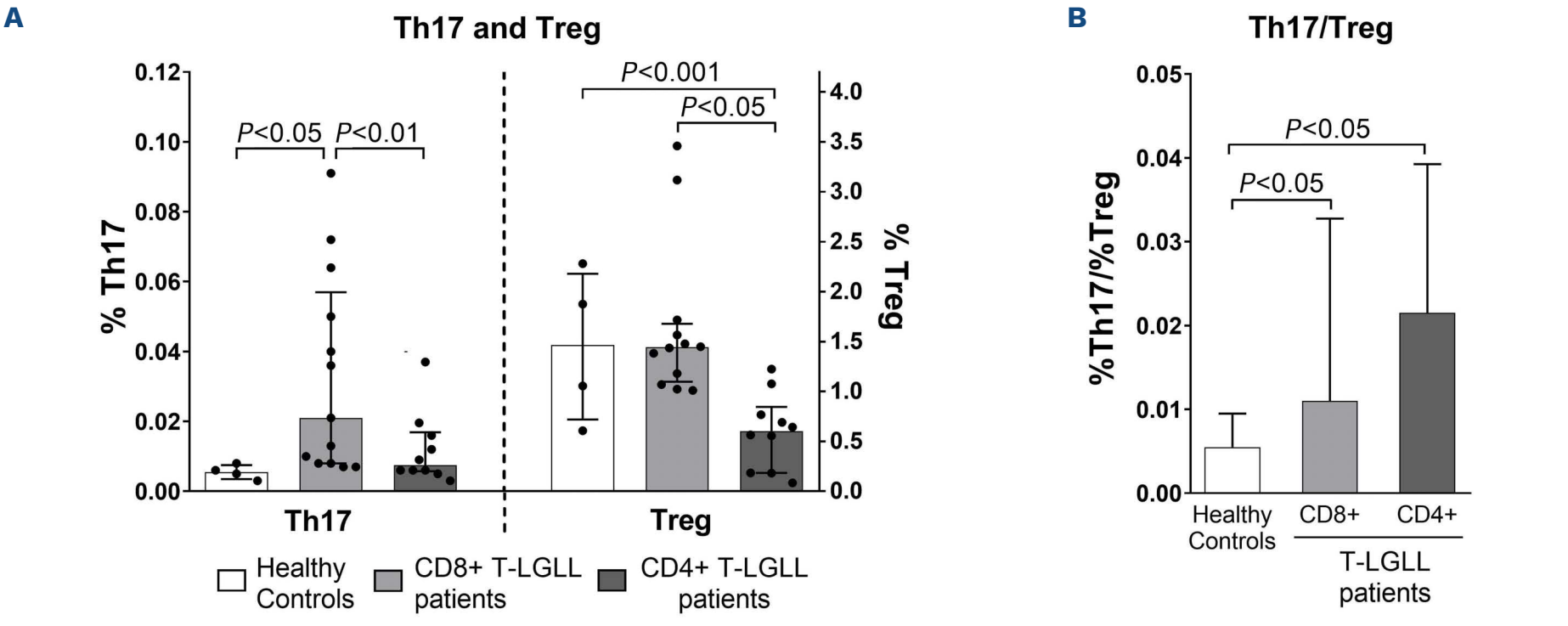

Figure 1. Distribution of Th17 and Treg cells in patients with T-cell large granular lymphocyte leukemia. (A, C) The percentages of Th17 and Treg were evaluated by flow cytometry. (B, D) The related Th17/Treg ratios were established mathematically. The Kruskal-Wallis test corrected for multiple comparisons was used for the analysis. Data are reported as histograms showing the median with interquartile range. (A, B) Patients with CD8$^+$ T-cell large granular lymphocyte leukemia (N=13) were characterized by an increase of Th17 cells, whereas Treg cells were reduced in CD4$^+$ cases (N=10). The Th17/Treg ratio was imbalanced for both groups in comparison to that of healthy controls (N=4). (C, D) An increased Th17 percentage characterizes CD8$^+$ wildtype (N=6) or *STAT3*-mutated (N=7) patients. Treg are reduced in CD4$^+$ wildtype (N=7) and *STAT5B*-mutated (N=3) patients, leading to a significant skewing of the Th17/Treg ratio in this group. T-LGLL: T-cell large granular lymphocyte leukemia; wt: wildtype; mut: mutated; pts: patients.

Results

Patients with CD8⁺ and CD4⁺ T-cell large granular lymphocyte leukemia are characterized by an unbalanced Th17/Treg ratio

A significant increase of the percentage of Th17 lymphocytes was found in CD8⁺ T-LGLL patients as compared with both HC and CD4⁺ cases (P<0.05 and P<0.01, respectively) (Figure 1A), while Treg percentage was significantly reduced in the CD4⁺ group compared to HC and CD8⁺ cases (P<0.001 and P<0.05, respectively) (Figure 1A). These imbalances led to an altered Th17/Treg ratio for both CD4 and CD8 T-LGLL patients related to HC (P<0.05) (Figure 1B). In terms of absolute count, the number of Th17 cells was significantly increased in CD4⁺ patients as compared to HC (P<0.01) (*Online Supplementary Figure S4A*), while the number of Treg was unchanged. This resulted in an altered Th17/Treg ratio for CD4⁺ T-LGLL (P<0.05) (*Online Supplementary Figure S4B*). Clustering patients according to *STAT3* and *STAT5B* mutations, increased Th17 cell percentages were documented in both CD8⁺ wt and *STAT3*-mutated patients (both P<0.05 *vs*. HC) while CD4⁺ cases (both *STAT5B*-mutated and wt) were characterized by a reduced percentage of Treg cells (Figure 1C). The evaluation of the absolute Th17 and Treg counts confirmed the relevant increase of Th17 cells (*Online Supplementary Figure S4C, D*). To note, the increased Th17 percentage and the skewed Th17/Treg ratio turned out to be a peculiar feature of CD8⁺ neutropenic patients (*Online Supplementary Figure S5A-C*). Since an abnormal distribution of Th17 and Treg is reported in autoimmune diseases,[22] which are often associated with CD8⁺ *STAT3*-mutated T-LGL expansions, we clustered our patients according to the incidence of autoimmune diseases. No differences in the distribution of Th17 and Treg cells were observed between patients with CD8⁺ *STAT3*-mutated T-LGLL with or without concomitant autoimmune diseases, also in terms of their related Th17/Treg ratio (*Online Supplementary Figure S5C-F*).

In further support of the results obtained, follow-up analysis showed that neutropenic CD8⁺ patients also maintained a higher percentage of Th17 cells compared to CD4⁺ cases at follow-up (53 months [interquartile range, 54.25-59.75]), whereas the CD4⁺ cases were characterized by a reduced percentage of Treg cells (*Online Supplementary Figure S8A-C*).

The distribution and counts of monocyte populations are altered in T-cell large granular lymphocyte leukemia

The distribution of peripheral blood monocyte populations of CD4⁺ T-LGLL patients displayed a pattern consistent with that of HC (Figure 2A, left panel; *Online Supplementary Figure S6A*, left panel). Conversely, in CD8⁺ T-LGLL the percentage of classical monocytes was significantly reduced (P<0.01 *vs*. HC and P<0.001 *vs*. CD4⁺ cases), with a slight increase of intermediate and non-classical subsets (Figure 2A, right panel). These alterations resulted specific to CD8⁺ *STAT3*-mutated patients. In particular, CD8⁺ *STAT3*-mutated cases displayed a significant reduction of classical monocytes (P<0.0001 *vs*. HC and CD8⁺ wt) and an increase of intermediate (P<0.05 *vs*. HC) and non-classical monocytes (P<0.01 *vs*. HC and P<0.05 vs. CD8⁺ wt) (Figure 2A; *Online Supplementary Figure S6A*). The alteration of monocyte percentages and their absolute counts strictly corresponded with the incidence of neutropenia (*Online Supplementary Figure S7A, B*). The percentage of classical monocytes was not influenced by the incidence of autoimmune diseases, while a significant reduction of their absolute count was observed (P<0.01) (*Online Supplementary Figure S7C, D*). The percentages of monocytes at follow-up were consistent with those observed at baseline (*Online Supplementary Figure S8D*).

Evaluation of monocytes using a cell blood count test showed that CD8⁺ *STAT3*-mutated patients were characterized by a reduced number of peripheral monocytes compared to CD8⁺ wt cases (P<0.05) (Figure 2B). Cell blood counts also demonstrated that monocyte percentage was inversely correlated with the leukemic clone count when considering the entire cohort of patients (r=-0.5, P<0.001) (Figure 2C). Consistent results were found when dividing patients into CD8⁺ and CD4⁺ cases (Figure 2D, E), specifically for wt cases (r=-0.55, P<0.05 for CD8⁺ wt and r=-0.84, P<0.01 for CD4⁺ wt) (*Online Supplementary Figure S6B-D*). In CD8⁺ *STAT3* and CD4⁺ *STAT5B*-mutated cases a significant correlation was not demonstrated (*Online Supplementary Figure S6E, F*).

Monocytes activate different cellular pathways among the T-cell large granular lymphocyte leukemia subgroups

Several pathways are involved in the activation of inflammatory signals in monocytes, including STAT3 and NFκB signaling.[23] Interestingly, a peculiar activation status of these two pathways was found among subgroups of T-LGLL patients (Figure 3). More in detail, STAT3 Tyr-705 phosphorylation was significantly increased in monocytes from CD8⁺ *STAT3*-mutated patients as compared to HC (P<0.05). This feature was also confirmed at the level of protein expression (P<0.001), with discrete statistical significance among the different subgroups (P<0.001 *vs*. CD8⁺ wt and *vs*. CD4⁺ wt cases; P<0.05 *vs*. CD4⁺ *STAT5B*-mutated cases) (Figure 3A). On the other hand, increased levels of phosphorylation of the NFκB subunit p65 were detected in CD8⁺ wt (P<0.05), CD4⁺ wt (P<0.001) and CD4⁺ *STAT5B*-mutated (P<0.001) patients compared to HC (Figure 3B). Monocytes from CD4⁺ *STAT5B*-mutated patients were characterized by a higher level of ERK phosphorylation as compared to those from HC (P<0.05) (Figure 3C). Representative western blot images are shown in *Online Supplementary Figure S9*.

Pattern of IL-6 and CCL5 expression in T-cell large granular lymphocyte leukemia

Since monocytes have been regarded as the principal source of IL-6,[13] in both T-LGLL patients and HC, we evaluated IL-6 expression levels within the different monocyte subsets by flow cytometry. Significantly higher IL-6 expression was observed in intermediate and non-classical monocytes compared with the classical ones in terms of percentage of IL-6 positive cells (P<0.0001 for both T-LGLL patients and HC) (Figure 4A panel on the left) and of

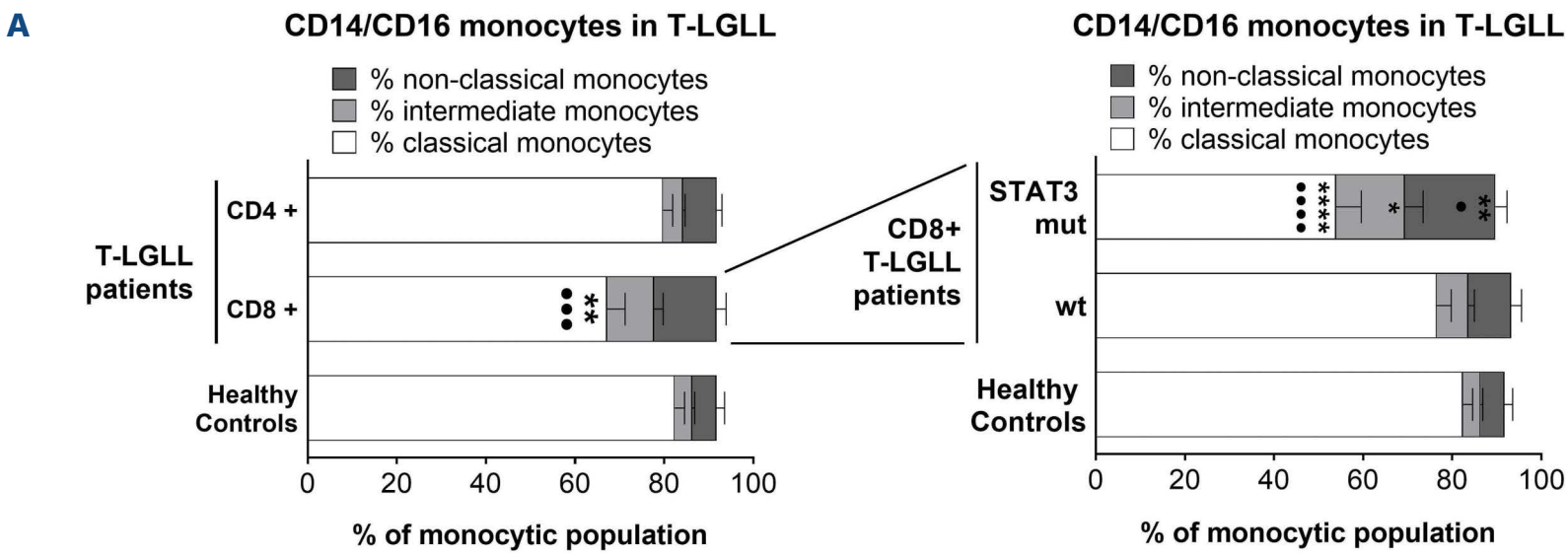

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Figure 2. Monocyte distribution in the peripheral blood of patients with T-cell large granular lymphocyte leukemia. (A) The percentages of classical, intermediate and non-classical monocytes were assessed by flow cytometry. Data are reported as histograms showing the mean with standard error and were analyzed by two-way analysis of variance. A reduction in classical monocytes was found in CD8$^+$ patients (N=17) in comparison to CD4$^+$ cases (N=12, •••P<0.001) and to healthy controls (N=5, $^{**}P$<0.01), as shown in the left panel. The imbalanced monocyte distribution resulted specific to *STAT3*-mutated cases (N=7) in comparison to CD8$^+$ wildtype (wt) ones (N=10, •) and healthy controls (*) (•••• and $^{****}P$<0.0001; $^{**}P$<0.01 • and $^{*}P$<0.05), right panel. (B) Monocyte counts in peripheral blood (PB), obtained from complete blood count values, are reported as histograms showing the median with interquartile range. Data were analyzed by the Kruskal-Wallis test and showed that the count was reduced in CD8$^+$ *STAT3*-mutated cases (N=16) in comparison to CD8$^+$ wt cases (N=17). Eleven CD4$^+$ wt and six *STAT5B*-mutated patients were also evaluated. (C-E) The correlation between the percentage of monocytes in peripheral blood and the leukemic clone count was evaluated in 50 patients with T-cell large granular lymphocyte leukemia (C), subsequently divided into 33 CD8$^+$ (D) and 17 CD4$^+$ (E) cases; Spearman analysis identified a significant inverse relation. T-LGLL: T-cell large granular lymphocyte leukemia; PB: peripheral blood; HC: healthy controls; mut: mutated; pts: patients; LGL: large granular lymphocytes.

levels of IL-6 expression (for T-LGLL patients, P<0.05 *vs*. intermediate and P<0.001 *vs*. non-classical monocytes), as previously described.[24] Intermediate monocytes were the cells with the highest IL-6 production (for T-LGLL patients, P<0.001 *vs*. non-classical monocytes) (Figure 4A panel on the right).

Although its precise cell source is still elusive, CCL5 expression was observed to be high in plasma and in patients' PBMC.[16] In our cohort of patients, leukemic LGL was pinpointed as the principal source of CCL5, as shown by higher mRNA level in comparison to its relative negative fraction (P<0.0001) (Figure 4B). Our results also indicated that CCL5 was quantitatively more expressed by CD8$^+$ T-LGLL as compared to CD4$^+$ T-LGLL and HC (P<0.05 and P<0.001, respectively)) (Figure 4B). In an extended cohort of patients, clustering patients according to *STAT3* and *STAT5B* mutations showed consistently higher CCL5 mRNA expression levels for both CD8$^+$ wt (P<0.05 *vs*. HC) and CD8$^+$ *STAT3*-mutated T-LGL (P<0.01 *vs*. HC) patients (Figure 4C).

Monocytes mediate survival of T-cell large granular lymphocyte leukemia cells through a cytokine interchange

To assess the impact of monocytes on the survival of the leukemic clone, *in vitro* cultures of PBMC with or without monocytes derived from the same blood sample from HC and T-LGLL patients were set up. When cultured with monocytes, leukemic LGL had a longer lifespan, at the different timepoints tested, in comparison to the LGL from HC (P<0.05) (Figure 5A). On the other hand, the survival of clonal LGL without monocytes was comparable to that of LGL from HC and significantly lower at 3 and 5 days in comparison to leukemic LGL cultured with monocytes (P<0.01) (Figure 5A). An extended cohort was used to get further insights into the role of monocytes in leukemic LGL survival among the different disease subgroups. When assessing the difference in percentage of apoptosis between PBMC without monocytes and PBMC with monocytes, an increased leukemic LGL mortality upon monocyte depletion, with respect to HC, was observed in all subsets with the only exception of the CD4$^+$ wt subgroup (P<0.001 for CD8$^+$ wt, P<0.01 for CD8$^+$ *STAT3*-mutated and P<0.001 for CD4$^+$ *STAT5B*-mutated cases) (Figure 5B).

Based on the above reported CCL5 overexpression in leukemic LGL and the high IL-6 expression on intermediate and non-classical monocytes (Figure 4), we hypothesized a putative monocyte-LGL communication loop. This suggestion is supported by the reduced expression of CCR5 on leukemic cells, previously described for neutropenic LGLL cases[18] and herein reported mainly for CD8$^+$ *STAT3*-mutated patients, and its variable expression on monocytes, with a trend to a higher expression on CD8$^+$ *STAT3*-mutated patients' monocytes (*Online Supplementary Figure S10A, B*). Patients' monocytes stimulated *in vitro* for 12 h with CCL5 did not express IL-6 mRNA (Figure 5C). However, a significantly higher IL-6 secretion was observed in monocytes obtained from T-LGLL patients in comparison to CCL5-treated monocytes from HC (P<0.05) (Figure 5D). Clustering for disease subsets did not reveal a subgroup specificity (*Online Supplementary Figure S10C*).

Discussion

In this study we provide evidence that a pro-inflammatory background plays a defined role in sustaining T-cell proliferation in T-LGL disorders. In particular, in CD8$^+$ *STAT3*-mutated LGLL patients we demonstrated a skewed Th17/Treg ratio and an abnormal distribution of monocyte populations characterized by reduced classical and increased intermediate and non-classical monocytes. These latter cells release high levels of IL-6 following CCL5 stimulation. We also demonstrated that T-LGLL patients' monocytes take advantage from abnormal intracellular signaling pathways, as compared to monocytes from HC. These data on the ability of the leukemic clone to shape the environment in favor of its own proliferation open a new paradigm in T-LGLL.

Accumulating evidence indicates that the intrinsic features of the LGL clone characterize the kinetics of the expanding population and contribute to define the disease subgroups.[1,3-5,11] Clustering T-LGLL patients according to immunophenotype and *STAT* mutational status allowed us

to demonstrate different types of signatures in relationship to the characteristics of non-leukemic cell populations, thus offering additional tools to separate disease subsets and suggesting peculiar pro-inflammatory mechanisms sustaining the pathogenesis of $CD8^+$ and $CD4^+$ T-LGLL. In fact, a skewed Th17/Treg ratio was a distinctive

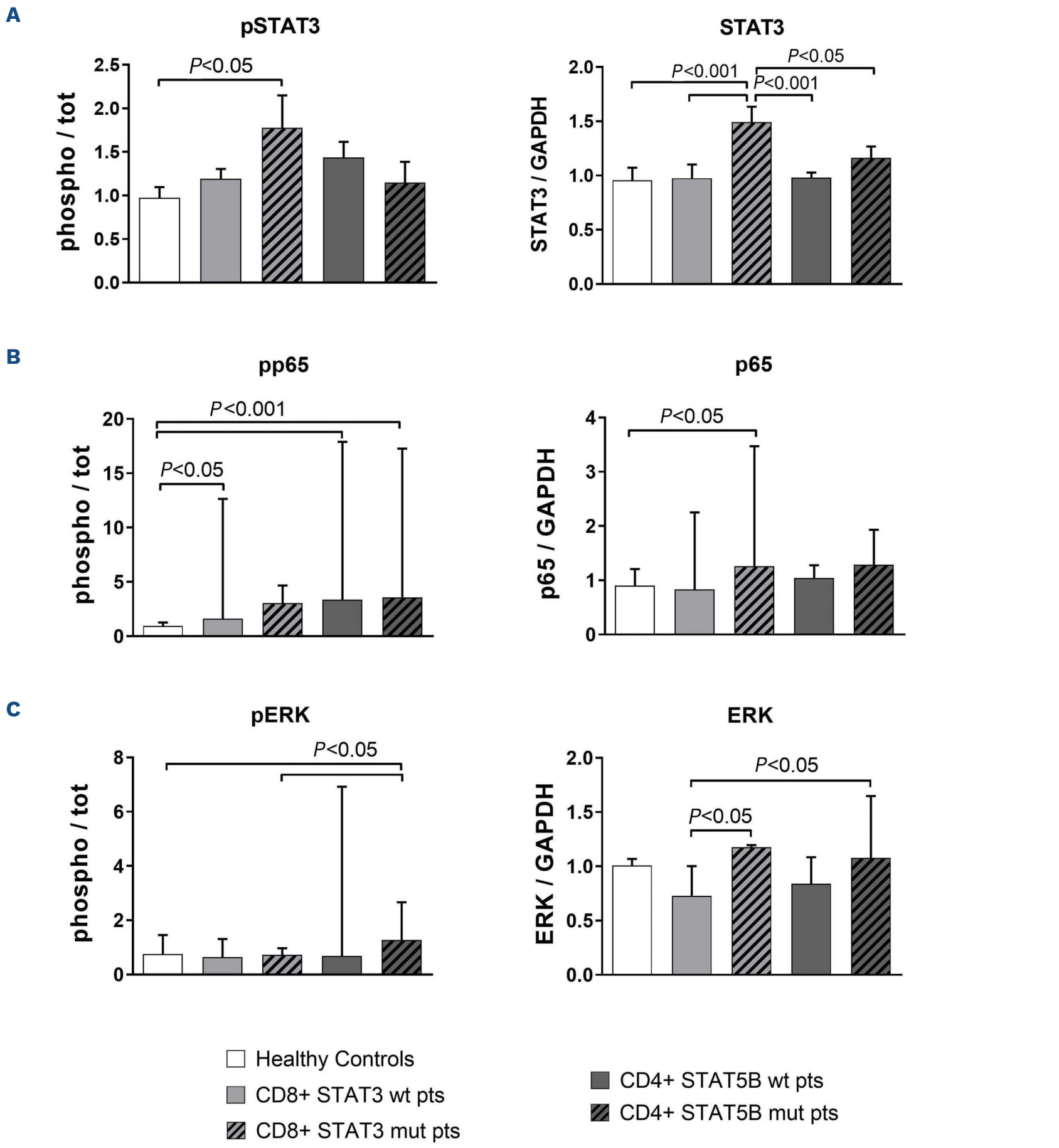


Figure 3. Activation of pathways in monocytes from patients with T-cell large granular lymphocyte leukemia. (A-C) Protein activation status and basal expression were assessed by western blot analysis in eight healthy controls and 30 patients with T-cell large granular lymphocyte leukemia (7 $CD8^+$ wildtype, 7 $CD8^+$ *STAT3*-mutated, 9 $CD4^+$ wildtype and 7 $CD4^+$ *STAT5B*-mutated). Densitometric values were subjected to analysis of variance or Kruskal-Wallis tests, according to the distribution of the data. Data are reported as histograms showing the mean with standard error (A) or median with interquartile range (B, C). (A) STAT3 (densitometric data were obtained by merging the optical density of the isoforms α and β) was more active in monocytes of $CD8^+$ *STAT3*-mutated cases, whereas (B) p65 was more phosphorylated in $CD8^+$ wildtype and $CD4^+$ patients. (C) $CD4^+$ *STAT5B*-mutated cases were characterized by a higher activation of ERK (densitometric data were obtained by merging the optical density of the isoforms 1 and 2). wt: wildtype; pts: patients; mut: mutated.

feature in CD8⁺ and CD4⁺ T-LGLL patients, CD8⁺ T-LGLL being characterized by a higher percentage of Th17 while a reduced percentage of Treg cells was detectable in CD4⁺ T-LGLL; these features were maintained during the patients' follow-up. The presence of a high percentage of Th17 cells was reported to stimulate CD8⁺ proliferation and increased cytotoxicity, suggesting a pro-proliferative role for these cells in CD8⁺ T-LGLL.[25,26] On the other hand, the reduction of Treg might be due to the marked plasticity of these cells, which not only downmodulate FoxP3 to become pro-inflammatory cells, but can also express Th1, Th17 or Th2 master regulators, turning out as helper T cell-like Treg.[27] All these findings might contribute to the pro-inflammatory environment needed for LGL proliferation. Looking at each T-LGLL subgroup in more detail, we identified that CD8⁺ *STAT3*-mutated and neutropenic pa-

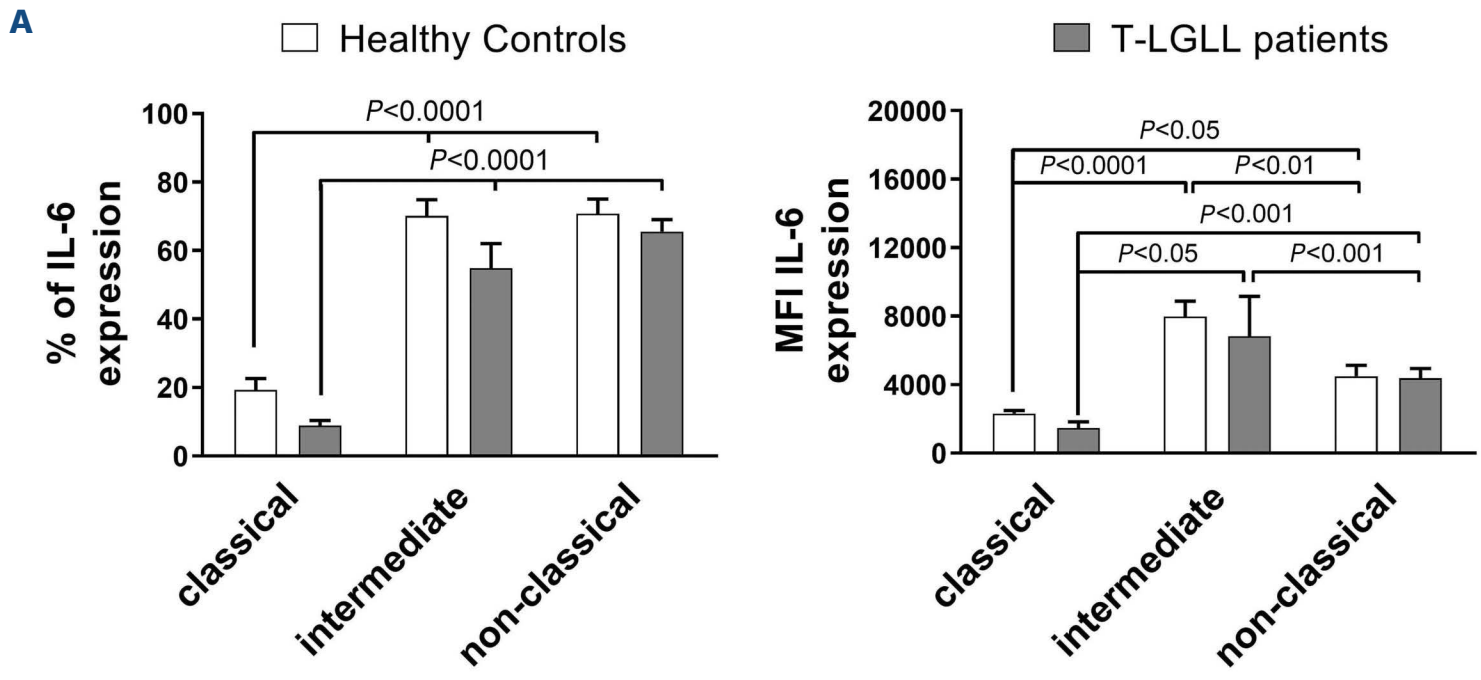
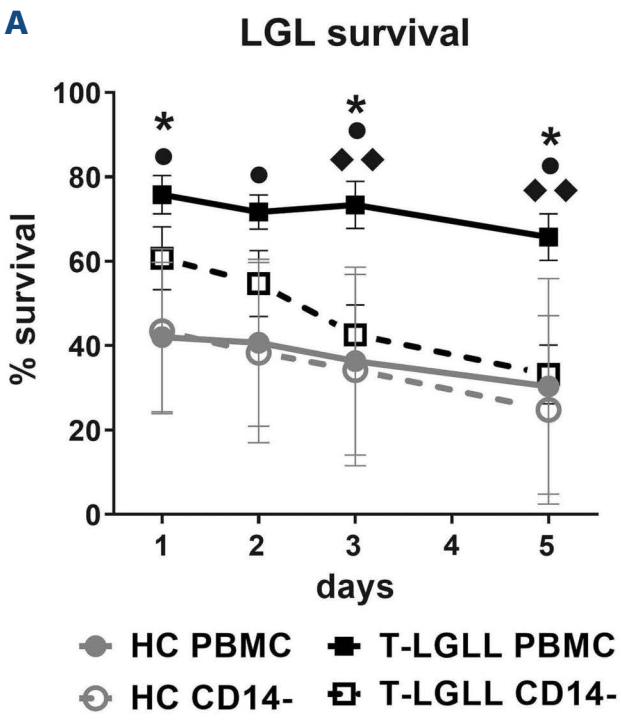
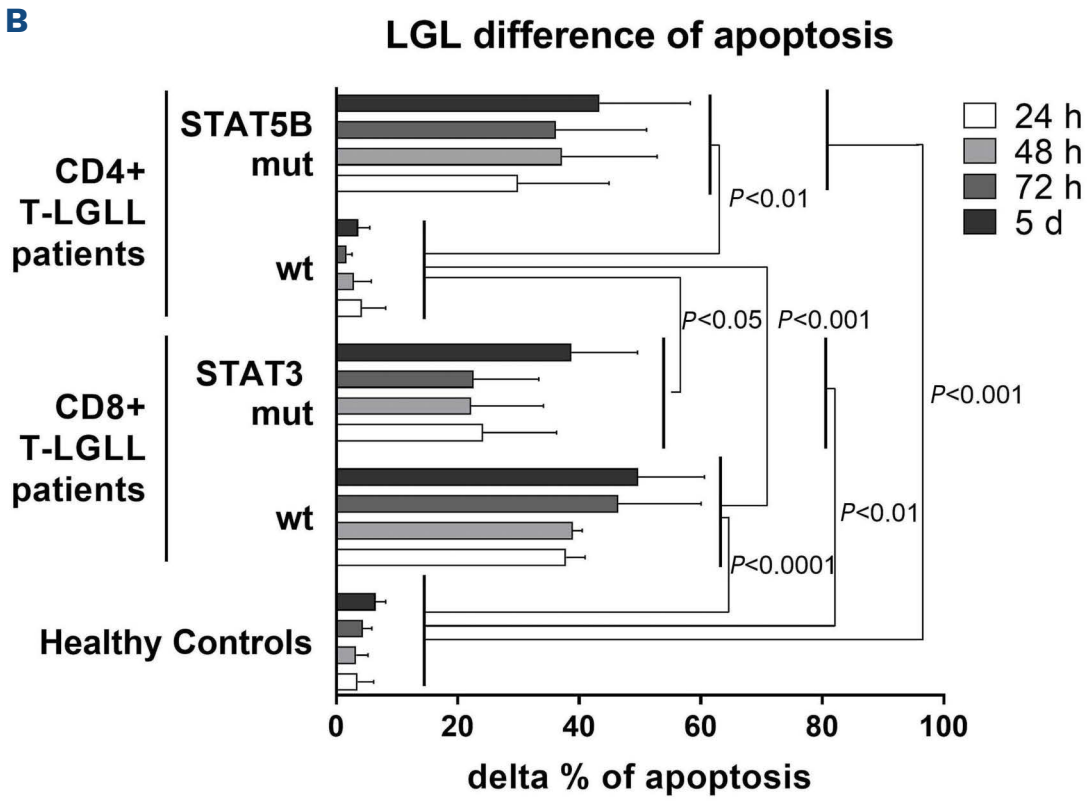
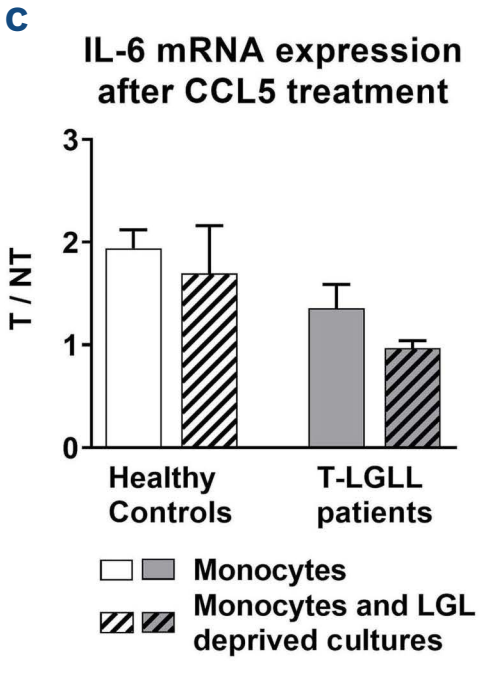


Figure 4. IL-6 and CCL5 expression in T-cell large granular lymphocyte leukemia. (A-C) Data are reported as histograms showing the mean with standard error. (A) The pattern of IL-6 expression, evaluated by flow cytometry in the monocyte subtypes in seven healthy controls and eight patients with T-cell large granular lymphocyte leukemia (T-LGLL), resulted significantly lower in classical monocytes for both groups after two-way analysis of variance; the decrease refers both to the percentage of IL-6-positive cells (left panel) and to the mean fluorescence intensity (right panel). (B) CCL5 expression was assessed by quantitative polymerase chain reaction and results are reported as arbitrary units (AU). Leukemic cells (LGL⁺) were immunomagnetically purified from CD8⁺ T-LGLL patients (N=6); higher levels of CCL5 were detected in LGL derived from CD8⁺ T-LGLL, by paired *t* test, as compared to the non-leukemic fraction (LGL⁻), than in LGL from healthy controls (N=3) and CD4⁺ cases (N=7). (C) The overexpression was confirmed in an enlarged cohort and was consistent in CD8⁺ wildtype (N=10) and *STAT3*-mutated (N=9) cases. Twelve CD4⁺ wildtype T-LGLL and nine CD4⁺ *STAT5B*-mutated patients were also evaluated. MFI: mean fluorescence intensity; AU: arbitrary units; pts: patients; LGL: large granular lymphocytes; HC: healthy controls; wt: wildtype; mut: mutated.







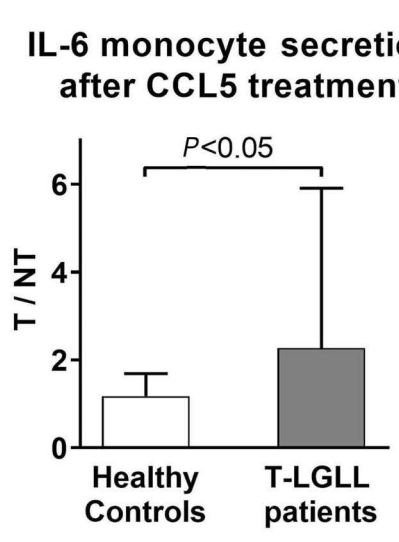


Figure 5. Evaluation of putative communications between monocytes and the leukemic clone in patients with T-cell large granular lymphocyte leukemia. (A) Monocytes from patients with T-cell large granular lymphocyte leukemia (T-LGLL) mediate the survival of the large granular lymphocytes (LGL) (evaluated by flow cytometry) *in vitro*. Patients' LGL (total N=10; 3 $CD8^+$ wildtype [wt], 4 $CD8^+$ *STAT3*-mutated and 3 $CD4^+$ *STAT5B*-mutated) cultured in peripheral blood mononuclear cells (PBMC) had a longer lifespan in comparison to healthy controls' LGL cultured in PBMC (HC PBMC, N=3; $^*P<0.05$), HC LGL cultured with PBMC immunomagnetically depleted of monocytes (HC $CD14^-$; •$P<0.05$) and leukemic LGL cultured without monocytes (T-LGLL $CD14^-$; ◆◆$P<0.01$). Data are reported as a scatter plot showing the mean with standard error. (B) In an extended cohort of patients, the difference of percentage of LGL apoptosis between *in vitro* cultures of PBMC without monocytes and PBMC with monocytes was determined by two-way analysis of variance and data are reported as histograms showing mean with standard error. A significant difference with respect to HC (N=6) was observed for $CD8^+$ T-LGLL patients, either *STAT3* wt (N=3) or mutated (N=7), and for $CD4^+$ *STAT5B*-mutated (N=4) cases; conversely, apoptosis levels for $CD4^+$ *STAT5B* wt patients (N=3) were consistent with those for HC. (C) Immunomagnetically purified monocytes and PBMC deprived of monocytes and LGL, obtained from ten patients (4 $CD8^+$ wt, 2 $CD8^+$ *STAT3*-mutated, 1 $CD4^+$ *STAT5* wt and 3 $CD4^+$ *STAT5B*-mutated) and five HC were cultured for 12 h under CCL5 stimulation (100 ng/mL). IL-6 induction of expression was evaluated by quantitative polymerase chain reaction and analyzed by *t* tests. Data are reported as histograms showing the mean ratio between treated and not treated conditions (T/NT) with the standard error. (D) Data obtained by enzyme-linked immunosorbent assay were analyzed by the Mann-Whitney test and are reported as histograms showing the median with interquartile range of T/NT values. A significantly higher IL-6 secretion was observed for T-LGLL patients (N=16 in total: 4 $CD8^+$ wt, 4 $CD8^+$ *STAT3*-mutated, 4 $CD4^+$ wt and 4 $CD4^+$ *STAT5B*-mutated) in comparison to HC (N=7). mut: mutated; h: hours; d: days.

tients are characterized by the highest number of "autoimmune-like" alterations, including an increased Th17/Treg ratio and the skewed monocyte distribution and count that are typically detected in autoimmune conditions.[19,20] These findings would suggest that concomitant autoimmune diseases are involved in shaping the peculiar environment detected in CD8$^+$ *STAT3*-mutated cases. The evidence that the same changes in cellular distribution were also detected in CD8$^+$ *STAT3*-mutated patients but without association with autoimmune diseases supports the concept that immune system alterations, identified in these patients, are actually an intrinsic trait of the disease and a potential concomitant autoimmune disease might amplify this feature.

Monocytes also contribute to the differences in the T-LGLL environment, since their number and population distribution differ across T-LGLL subtypes. In particular, the inverse relationship among leukemic clone burden and monocyte percentage resulted stronger for CD8$^+$ wt and CD4$^+$ wt cases; going into detail, CD8$^+$ *STAT3*-mutated patients were characterized by an increased count of pro-inflammatory monocytes notwithstanding the general reduction in monocyte count. Despite being reduced during the expansion of the leukemic clone, monocytes resulted relevant for T-LGLL pathogenesis mostly because of their altered activation status. In fact, monocytes from CD8$^+$ *STAT3*-mutated patients were found to be characterized by increased STAT3 phosphorylation, a well-known marker of environmental and tumor cell hyperactivation which has been reported to accelerate the development of cancerous cells.[28,29] This is consistent with our previous data demonstrating that both intrinsic and extrinsic mechanisms contribute to maintain the JAK/STAT pathway aberrantly activated in T-LGLL patients.[13] On the other hand, these findings further highlight the central role of STAT3 activation in T-LGLL pathogenesis. In fact, monocytes were proven to contribute to leukemic cell survival in the majority of T-LGLL subsets, i.e., CD8$^+$ (both *STAT3*-mutated and wt) and CD4$^+$ *STAT5B*-mutated cases. In fact, monocytes derived from patients not *STAT3*-mutated (i.e., CD8$^+$ wt and CD4$^+$), were found to be equipped with strong p65 activation. The NFκB and ERK hyperactivation we detected in monocytes from CD4$^+$ *STAT5B*-mutated patients suggests that NFκB signaling mediates inflammation through cellular senescence in non-classical monocytes.[24] Together with the identification of a higher cell-to-cell contact between CD8$^+$ leukemic T-LGL and monocytes, these findings indicate that pro-inflammatory cells could generate discrete settings specific for each T-LGLL subgroup, even within the indolent form of T-LGLL.[30] The high IL-6 release by intermediate and non-classical monocytes and the overexpression of CCL5 by CD8$^+$ leukemic cells lead to the suggestion that monocytes mediate T-LGL survival through the interchange of cytokines, especially IL-6 and CCL5. This mechanism is likely to be particularly active in symptomatic patients, since they have high IL-6-secreting monocytes. This adds importance to the role of IL-6 in the pathogenesis of T-LGLL, which was previously described by our group[13] and recently linked to the efficacy of alemtuzumab in T-LGLL.[31] Moreover, we show a putative pathogenic mechanism of CCL5 in T-LGLL, since its expression was recently associated with CD8$^+$ *STAT3*-mutated LGL with high NKG2D[32] and consistently herein demonstrated to be more expressed in CD8$^+$ clones than in controls and CD4$^+$ cases. Nonetheless, Th17 cells, being increased in CD8$^+$ *STAT3*-mutated cases, are likely to be involved in the monocyte-LGL loop; it has been reported that this T-cell subset could stimulate CCL5 expression in cytotoxic T lymphocytes.[33,34] Accordingly, we noticed a tendency of CCL5 expression induction after stimulation of purified LGL with IL-17A for 24 h (*Online Supplementary Figure S11B*), suggesting the rationale for the use of IL-17 inhibitors in the treatment of T-LGLL patients, as recently proposed.[35]

We believe that the exchange of IL-6 and CCL5 between monocytes and leukemic cells is one of several ways of communication between leukemic cells and environmental cells. Many different cytokines have already been described as relevant in T-LGLL, such as PDGF, IL-18 and IL-15, this last recently involved in the accumulation of CD8$^+$ LGL cells in models of *STAT3* germinal gain-of-function mutation.[16,17,32,36] For these reasons, a better definition of the pro-inflammatory mechanisms, possibly also considering the bone marrow microenvironment, might clarify why, in the presence of different immunophenotypes and *STAT* somatic mutations, LGL leukemic clones can shape a peculiar environment.

Bone marrow analysis is nowadays not currently required in clinical practice in T-LGLL patients.[1] This constraint causes a limitation to our results and their interpretation, the evaluation of cell populations being restricted to the peripheral blood. It is, however, plausible that the changes we observed in peripheral blood reflect what is occurring in tissue microenvironment(s). In the bone marrow microenvironment, dendritic cells are in close contact with LGL and they have been claimed to represent the setting in which the putative antigen presentation takes place.[37] Moreover, it has also been suggested that dendritic cells may be the relevant presenting cells that trigger the clonal proliferation and maintain LGL expansion by cytokine production, in particular IL-15,[38] a cytokine that has been found to induce T-LGLL in transgenic mice.[39]

This work unveils the role of non-leukemic cells in T-LGLL and demonstrates that T-LGLL subgroups can be further differentiated not only according to biological hallmarks of leukemic cells and/or clinical aspects but also considering environmental features. The potential interactive pathway between leukemic cells, monocytes and Th17

lymphocytes we described in the symptomatic and treatment-requiring patients (i.e., mainly $CD8^+$ *STAT3*-mutated) might pave the way for the design of new therapeutic strategies, in view of the evidence that several anticancer drugs have been shown to reprogram monocytes in a tumor microenvironment.[40] Furthermore, since the communication loop between *STAT3*-mutated leukemic cells and monocytes might be mediated by the activation of CCR5, with the involvement of Th17 cells, the use of CCR5 or Th17 antagonists might represent an interesting option for the treatment of T-LGLL patients. The environmental alterations identified in non-symptomatic patients indicate that also in these cases leukemic T-LGL rely on pro-inflammatory stimuli, thus giving rise to new questions on the role of the microenvironment in the pathogensis of T-LGLL and its targetability for therapeutic purposes.

Disclosures

No conflicts of interest to disclose.

Contributions

CV, GS, and RZ conceived the study. CV, VRG, ER, AT, and GC purified the cells from patients. CV performed the experiments for the study. CV, SC, and VT performed the flow cytometry analysis. GB, MF, LT, and RZ provided the patients' clinical features. CV wrote the paper. RZ and GS critically reviewed the manuscript. All authors revised the manuscript.

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Data-sharing statement

Data are available upon request.

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