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LGL leukemia: from pathogenesis to treatment

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Abstract

Large granular lymphocyte (LGL) leukemia has been recognized by the WHO classifications among mature T cell and NK cell neoplasms. There are 3 categories: chronic T cell leukemia and NK cell lymphocytosis which are similarly indolent diseases characterized by cytopenias and autoimmune conditions as opposed to aggressive NK cell LGL leukemia. Clonal LGL expansion arise from chronic antigenic stimulation which promotes dysregulation of apoptosis, mainly due to constitutive activation of survival pathways including Jak/Stat, MapK, Pi3k-Akt, RasRaf-1, MEK1/ERK, sphingolipid, and NFκB. SoCs3 down regulation may also contribute to Stat3 activation. IL15 plays a key role in activation of leukemic LGL. Several somatic mutations including Stat3, Stat5b, and TNFAIP3 have been demonstrated recently in LGL leukemia. Since these mutations are present in less than half of the patients they cannot completely explain LGL leukemogenesis. A better mechanistic understanding of leukemic LGL survival will allow future consideration of a more targeted therapeutic approach than the current practice of immunosuppressive therapy.
Introduction

Initially described in 1985, Large Granular Lymphocyte (LGL) leukemia belongs to the rare chronic mature lymphoproliferative disorders of the T/NK lineage. Two subtypes of LGL disorders were proposed in 1993: T-LGL leukemia and aggressive NK cell leukemia. The WHO recognized this classification scheme in 2001. Chronic NK-cell lymphocytosis was identified in 2008 as a provisional entity to differentiate it from the much more aggressive form of NK-cell leukemia. The most recent WHO version did not modify this classification scheme but did highlight discovery of Stat mutations described in 2012 (table 1). T-LGL leukemia and chronic NK cell lymphocytosis share the same clinical and biological presentation as well as treatment options. Pathogenesis of the disease is dominated by a clonal expansion of LGL resistant to activation induced cell death (AICD) due to constitutive survival signaling. This review will describe topics concerning diagnosis, pathogenesis, current and future therapy of this rare disease.

Epidemiology

LGL leukemia accounts for 2–5% of chronic lymphoproliferative disorders in North America and Europe and up to 5–6% in Asia. Recently, the overall age-standardized incidence based on the national Dutch registry has been reported as 0.72 per 1,000,000 person-per year. The incidence of LGL leukemia does not differ between male and female. Indolent T-LGL leukemia is the most frequent form of the disease representing around 85% of the cases whereas chronic NK cell lymphocytosis is estimated at less than 10% of cases. Aggressive NK LGL leukemia is mainly seen in Asia, comprising less than 5% of the LGL disorders. It affects younger patients and is associated with EBV infection: the prognosis of this rare entity is very poor due to refractoriness to chemotherapy.

Diagnosis

A definite diagnosis of LGL leukemia requires finding evidence of an expanded clonal T or NK cell LGL population.
Cytology

The first step of diagnosis is based on identification of increased numbers of circulating LGL. Initially, a LGL count $> 2 \times 10^{9}/l$ (normal value: $< 0.3 \times 10^{9}/l$) was mandatory\textsuperscript{13,14} but a lower number may be compatible with the diagnosis if these cells are clonal and the patient displays other clinical or hematological features such as rheumatoid arthritis or cytopenias. Indeed, it has been found that some patients with relatively low LGL counts (even less than $1 \times 10^{9}/l$) have a clonal disorder.\textsuperscript{9,14,15,16} Leukemic LGL are easily identified on blood smears by their specific morphology; however, they are not cytologically distinguishable from normal reactive cytotoxic lymphocytes. They display a large size (15-18$\mu$), an abundant cytoplasm containing typical azurophilic granules and a reniform or round nucleus with mature chromatin (Fig 1 A). Blood smears must be examined carefully in cases of normal lymphocyte counts and in rare cases in which the clonal lymphocytes do not present with typical LGL morphology. LGL excess in bone marrow (> 10% LGL) is detected in the majority of cases.\textsuperscript{12}(Figure 1B)

Immunophenotyping

T-LGL leukemias show a constitutive mature post-thymic phenotype. In the vast majority of cases, T-LGL leukemia shows a CD3+, TCR $\alpha\beta$, CD4-, CD5\textsuperscript{dim}, CD8+, CD16+, CD27-, CD28-, CD45RA-, CD27-, CD57+ phenotype which represents a constitutively activated T cell phenotype (Fig 2A).\textsuperscript{17,18,19} CD3+/CD56+ T-LGL leukemias may have a more aggressive behavior associated with Stat5b mutations.\textsuperscript{20,21} A rare subset of LGL leukemia is CD4+ with or without coexpression of CD8. Patients with this LGL leukemia subtype almost never have cytopenias, splenomegaly or autoimmune phenomena\textsuperscript{22,23}, and clonal LGL seem to be driven by CMV.\textsuperscript{24} Recently, it was found that Stat5b mutations are frequent in this LGL subtype.\textsuperscript{25}

T-LGL leukemic cells are characterized by a terminal-effector memory phenotype defined by the expression of CD45RA and lack of CD62L expression.\textsuperscript{26} Leukemic LGL constitutively express IL2 R$\beta$ (p75, CD122) but not IL2 R$\alpha$ (p55, CD25).\textsuperscript{27,28} Few cases are TCR $\gamma\delta+/CD4-/CD8-$.\textsuperscript{29,30}
NK-LGL leukemia and NK-LGL lymphocytosis are characterized by the following phenotype: CD2+/sCD3-/CD3ε+/TCRεβ-/CD4-/CD8+/CD16+/CD56+ (Fig 2B). Fas (CD95) and Fas-Ligand (CD178) are strongly expressed in LGL leukemia. Restricted KIR expression is often seen in both T and NK LGL leukemia.

**Clonality**

Evidence of T-LGL clonality is routinely assessed using TCR γ-PCR analyses. Deep sequencing of TCR has demonstrated a restricted diversity of TCR repertoire. Vβ TCR gene repertoire analysis can also be ascertained using flow cytometry (FCM) and serves as presumptive evidence for clonality. The current Vβ MoAbs panel covers 75% of the Vβ spectrum with a high correlation between Vβ FCM and TCRγ-PCR results. Fluctuations in clonal dominance, as determined by CDR3 sequencing, are seen in up to one third of patients. It is difficult to assess clonality of NK LGL as these cells do not express TCR. Restricted expression of activating isoforms of killer immunoglobulin-like receptors (KIR) has been utilized as a surrogate marker for a monoclonal expansion (Fig. 2B).

Clonal chromosomal abnormalities have been reported in a few cases of LGL leukemia.

**Molecular findings**

Constitutive activation of Stat3 in all LGL leukemia patients was first discovered in 2001. In 2012, common somatic gain-of-function Stat3 mutations were demonstrated in 28% to 75% of T-LGL leukemia and 30% to 48% of NK LGL lymphocytosis. These differences may be due to the sequencing technique and the selection of patients. Detection of identical Stat3 mutations in both T and NK subtypes suggests a unifying pathogenesis for these similar disorders. Mutations are primarily located in exons 20 and 21 encoding the Src homology 2 (SH2) domain, which drives the dimerization and activation of the Stat protein. D661 and Y640 account for two thirds of mutations. Activating mutations outside the SH2 domain are rarely detected. Such mutations are located in the DNA-binding and coiled-coil domain of Stat3. Utilization of deep sequencing has demonstrated presence of multiple subclones containing different Stat3 mutations in distinct LGL
populations in individual patients. These findings suggest a potential need for sequencing the entire Stat3 gene. Whether or not Stat3 mutations are correlated with specific clinico-biological features remains uncertain and subject of ongoing research. The ECOG prospective clinical trial suggested that a particular Stat3 mutation, Y640F, predicted response to initial therapy with methotrexate. The first evidence of Stat5b mutations in human disease was discovered in LGL leukemia, but this mutation is not frequent (2%). The N642H mutation in particular was associated with a more aggressive disease and a unusual CD3+CD56+ phenotype.

Marrow Features
The diagnosis of LGL leukemia as discussed above is readily established using blood studies, so that marrow aspirate/biopsy is not routinely recommended as part of initial evaluation. However, marrow evaluation can be helpful when the diagnosis is not straight-forward (eg LGL count not increased). Such studies are also of value when considering other diseases that are part of the differential, including MDS or aplastic anemia or when considering the diagnosis of pure red cell aplasia (PRCA) as potential etiology of profound anemia in patients with LGL leukemia.

Individual or small clusters of LGLs may be sometimes identified. They are difficult to identify as they mimick granulocytic or monocytic precursors. As LGL infiltration of marrow is often a subtle finding, trephine biopsy with immunohistochemistry is recommended. A “grey zone” where the diagnosis of LGL leukemia is not certain, should be considered in cases of low LGL count, or even lymphopenia associated with unexplained cytopenia in which the diagnosis can be easily missed (Figure 3).

Classical histological features are depicted in figure 4. Clusters of eight CD8+/TiA1+ cells or six Granzyme B+ lymphocytes are considered as characteristic histopathologic findings of LGL leukemia, and support this diagnosis in uncertain cases. Very close topographic distribution between dendritic cells and LGL in patient marrow samples have been shown, suggesting constant antigen presentation. Trilineage hematopoiesis in bone marrow biopsies of LGL patients is normal or increased in the majority of cases. Apoptotic figures, increased macrophages, and eosinophilia have been described, suggesting an underlying dysmyelopoiesis. In neutropenic patients, a typical finding is decrease in granulocyte precursors and left-shift maturation. However, degree of marrow infiltration by LGL
does not correlate with degree of cytopenia. Increase of erythroid precursors is described in 30-40% of the cases. Reticulin fibrosis is usually present, ranging from grade II or III in about 50-60% of the cases.

**Clinico-biological features**

LGL leukemia principally affects elderly people with a median age of 60 years. Very rare pediatric cases have been reported and less than 25% of adult patients are younger than 50 years old. About one third of patients are asymptomatic at the time of diagnosis. Initial presentation is mainly related to neutropenia and includes recurrent oral aphthous ulcerations, fever secondary to bacterial infections. These infections typically involve skin, oropharynx and perirectal areas, but severe sepsis may occur. However, some patients may have profound and persistent neutropenia without any infections over a very long period of time. The frequency of recurrent infections varies in different series from 15 to 39%. Fatigue and B symptoms are observed in 20 to 30% of cases. Splenomegaly is reported with a frequency varying from 20 to 50% and lymphadenopathy is rare. Half of the patients present with lymphocyte counts between 4 \times 10^9/L and 10 \times 10^9/L, and the LGL count usually ranges from 1 to 6 \times 10^9/L. A lower LGL count (0.5 to 1 \times 10^9/L) may be observed in 7% to 36% of cases. Severe neutropenia (<0.5 \times 10^9/L) and moderate neutropenia (<1.5 \times 10^9/L) are observed in 16% to 48%, and 48% to 80% of cases, respectively. Anemia is frequent; transfusion dependent patients are observed in 10 to 30% of cases. PRCA occurs in 8 to 19% of the cases and moderate thrombocytopenia is observed in less than 25% of patients. While not routinely performed, increased soluble Fas-Ligand (sFas-L) is a good surrogate marker of LGL leukemia. We and others showed that LGL leukemia patients had increased serum levels of interferon α2, MCP-1 (an attractive factor of monocytes, T and NK cells to sites of inflammation), EGF, IL6, IL8, and IL18. High β2 microglobulin level is observed in 70% of cases. Rheumatoid factor and antinuclear antibody are detected in 60% and 40% of patients, respectively. Serum protein electrophoresis usually shows polyclonal hypergammaglobulinemia due to increased IgG and/or IgA subclasses. Defects in downregulation of Ig secretion in
LGL leukemia could partly explain the development of autoantibodies and clonal B-cell malignancies observed in this disease.\textsuperscript{64} The associated comorbid conditions are reported in table 2. Rheumatoid arthritis (RA) is the most common associated disease, occurring in 10 to 18 % of patients.\textsuperscript{7,18,65,66} Systemic lupus erythematosus (SLE), Sjogren’s syndrome, autoimmune thyroid disorders, coagulopathy, vasculitis with cryoglobulinemia and inclusion body myositis have occasionally been reported.\textsuperscript{65,67,68,69} Pulmonary artery hypertension (PAH) has been observed.\textsuperscript{70} Association with myeloid malignancies, and bone marrow failure syndromes, i.e Aplastic anemia (AA), paroxysmal nocturnal hemoglobinuria (PNH) and myelodysplasia (MDS), is also reported.\textsuperscript{71,72,73} Recurrent Stat3 mutations in de novo AA and MDS suggest similar pathogenesis.\textsuperscript{42} Efficacy of immunosuppressive treatments directed against T lymphocyte-mediated immune response is a strong argument for a common role of auto reactive T cells in all of these diseases.

**Pathogenesis of LGL Leukemia**

A model of LGL leukemia pathogenesis is shown in Figure 5. LGL leukemia is at the intersection of a clonal lymphoproliferative disease, chronic inflammation, and autoimmunity. We hypothesize that an unknown antigen is the initial activating event, resulting in oligoclonal LGL expansion. Such chronic and persistent antigen exposure leads to Stat3 activation and emergence of a dominant clone. A shift from oligoclonal to clonal dominance is supported by serial studies of T cell repertoire utilization.\textsuperscript{37} Somewhat surprisingly, clonal drift was observed with emergence over time of a different Vbeta clone in 37% of patients.\textsuperscript{37} We speculate that clonal drift results from emergence of LGL clones recognizing different epitopes/peptides of the same chronic antigen. This theory could also explain the observation of clonal T cell LGL populations seen in patients with chronic NK lymphocytosis as well as emergence of a new NK clone in patient with established T cell LGL leukemia.\textsuperscript{74} LGL leukemia is characterized by profound dysregulation of the normal process of AICD. The fundamental pathogenic feature of LGL leukemia is activation of a survival network which keeps the leukemic LGL alive and functioning as killer cells.\textsuperscript{75} The central hub of this survival network is Stat3. We hypothesize that activating mutations of Stat3 support clonal dominance by causing a higher level of
transcription of survival components. This theory is supported by the observation that STAT3 mutations are correlated with a larger clone size in LGL leukemia patients.\(^4\) Disease manifestations such as cytopenias and autoimmune diseases then result from production of proinflammatory cytokines mediated by STAT 3 regulation as well as attack on marrow and joints by the STAT 3-activated LGL. The key molecular signaling components in LGL leukemia are depicted in Figure 6. Key features of the model are further summarized below.

An initial viral antigenic stimulation
LGL leukemic cells represent an expanded population of an effector memory cytotoxic T cells, suggesting chronic antigen stimulation.\(^2\) A role for HTLV retroviral infection has been suggested primarily by serologic studies demonstrating cross-reactivity to HTLV epitopes. Such seroreactivity is directed towards the envelope protein BA21 and is seen in 30 to 50% of the patients. However, there is no definite evidence for HTLV-I infection in LGL leukemia patients\(^7\) while HTLV-2 has been described only occasionally.\(^8\)

Two cases of LGL leukemia associated with B indolent lymphoma were reported in patient infected with HCV infection. Both were successfully treated by antiviral therapy. This underlies a potential link between HCV chronic infection and LGL leukemia.\(^9\) Recently Sandberg et al analyzed CDR3 \(\beta\) and \(\alpha\) chain in 26 T CD8+ \(\alpha\beta\) TCR LGL patients and found heterogeneity in CDR3 region, suggesting that a unique antigen driving LGL leukemia may be unlikely.\(^10\)

Initiating role of IL-15 and PDGF
IL15 pro-inflammatory cytokine and PDGF play a crucial role in LGL leukemia expansion by promoting NK cell or leukemic LGL survival.\(^11,12\) IL15 receptor-\(\alpha\) -/- mice display a NK cell and CD8+ memory T cell defect. LGL cell growth is dependent of IL15 and IL15\(\alpha\) chain mRNA was detectable in LGL purified cells.\(^13,14\) In a network model of LGL leukemia survival signaling, IL15 and PDGF were predicted to be the master activation switches necessary for leukemic LGL survival.\(^11\) IL15 has also been implicated in LGL leukemogenesis using a unique IL15 transgenic mouse model demonstrating an increase of global DNA methylation level in LGL cells.\(^15\) Activation of survival pathways by PDGF occurs through an autocrine loop in leukemic LGL.\(^16\)
Constitutive activation of Jak-Stat3 signaling pathway
Constitutive activation of Stat3 (pStat3), in our opinion, plays the fundamental pathogenic role in LGL leukemia, as the transcriptome of LGL leukemia patients is that of Stat3-regulated genes. In vitro inhibition of Stat3 by STA-21 restores apoptosis of LGL cell independent of Stat3 mutational status. This finding implies that Stat3 mutation is not itself mandatory to explain LGL clonal expansion. It raises the question of mechanism of Stat3 activation in unmutated patients. The pro-inflammatory cytokine IL6 is able to activate Jak-Stat3 pathway and high amount of IL6 is observed in LGL patients.63 Inhibition of this cytokine restores LGL apoptosis. Dendritic cells could trigger the clonal proliferation and maintain LGL expansion by IL6 production. Because SOCS3 transcription is induced by IL-6 through P-Stat3, SOCS3 was postulated to be high in LGL leukemia. Surprisingly, Teramo et al, showed that SOCS3 mRNA and protein level was significantly decreased in LGL leukemia. Demethylation agent restored SOCS3 expression in leukemic LGL and consequently decreased Stat3 and McI-1 expression. No mutation or methylation of SOCS3 promoter has been found to explain this down regulation.63 Although Stat3 activation plays the most important pathogenenic role, multiple other cell survival pathways are dysregulated and these are briefly described below.

Resistance to Fas/Fas ligand mediated apoptosis
Leukemic LGL are resistant to Fas mediated apoptosis.31 However, apoptosis is restored using IL2 stimulation suggesting an inhibition of this pathway and not a complete defect. Moreover, soluble Fas (sFas), detected in LGL patient sera, acts as a decoy receptor able to inhibit Fas-dependent apoptosis.61 Decreased level of an inhibitory protein named c-FLIP is found in leukemic LGL contributing to DICS formation defect. This protein blocks the initial event of Fas-mediated apoptosis through FADD (Fas-associated protein with death domain) and caspase 8 recruitment.26

Ras-Raf-1-MEK1-ERK pathway
Overactive Ras plays a role in the survival signaling in LGL leukemia. Constitutive activation of Ras and extracellular-regulated kinase (ERK) was found in NK-LGL leukemia and G12 KRAS mutation was detected in LGL leukemia cell line.90
Blockade of either ERK or Ras activity may restore Fas sensitivity in leukemic LGLs.91

**Dysregulation of PI3K/Akt pathway**
Activated Akt is observed in leukemic LGL. Pro-inflammatory proteins RANTES, IL-18 and MIP-1b which activate PI3K pathway, are upregulated in LGL patients.62
Increased activity of the PI3K-AKT signaling axis has been found in T-LGL cells and participated in apoptosis inhibition.92

**Activation of NFκB pathway**
Leukemic LGL exhibit higher level of c-Rel (a member of NFκB member family) and high NFκB activity. NFκB inhibition induces leukemic LGL apoptosis. It has been shown that NFκB acts downstream of the PI3K-AKT pathway to prevent apoptosis through Mcl-1 independently of Stat3.75 Recently, a recurrent non-synonymous mutation in the gene encoding a NFκB signaling inhibitor, TNFAIP3, was found in three patient out of 39 patients (8%), underlying the important role of NFκB activity in LGL leukemia.93

**Dysregulation of sphingolipid rheostat in LGL leukemia**
Molecular expression profile analysis in LGL leukemia shows a predominant expression of pro-survival sphingolipids like S1P.94 Sphingosine kinase 1 (SphK1) which converts sphingosine into SP1 is increased in LGL leukemia and SphK1 inhibition leads to leukemic LGLs apoptosis. SP1 binding to SP1R activates pro survival signal through ERK1/2 signaling.95 Moreover, expression of S1P receptor, mainly S1PR5, is increased in LGL leukemia.96,97

**PROGNOSIS**

T and chronic NK-LGL leukemia are considered indolent diseases. The vast majority of patients will eventually need treatment at some point during disease evolution. Disease related deaths are mainly due to severe infections which occur in less than 10% of the patient population.10,15,98 Overall survival at 10 years is close to 70%.
Conversely, the prognosis of aggressive NK LGL leukemia is very poor since patients are refractory to treatment. 11,12

THERAPY

Treatment options are similar for T-LGL leukemia and chronic NK-cell lymphocytosis. Indications for treatment include severe neutropenia (ANC < 0.5 x 10^9/L), moderate neutropenia (ANC >0.5 x 10^9/L) associated with recurrent infections, symptomatic or transfusion dependent anemia and associated autoimmune conditions requiring therapy^9. Standard treatment of LGL leukemia is immunosuppressive therapy but such treatment recommendations are primarily based on small retrospective series. The most clinical experience has been reported using low-dose methotrexate, cyclophosphamide, and cyclosporine as single agents (see below).

Supportive therapy

Supportive care could be considered for patients suffering from anemia or neutropenia using EPO or G-CSF, respectively. However, such treatment does not address the underlying illness. G-CSF delivered as a single agent may be effective in rapidly increasing ANC. This could be of some value in the setting of patients with episodes of severe febrile neutropenia in which a rapid neutrophil response would be desirable. However, G-CSF is not effective in all LGL leukemia patients. Of note, G-CSF may induce exacerbation of splenomegaly and articular symptoms.99,100 Therapy with EPO in LGL leukemia patients has been reported only infrequently and with disappointing results.15

First line therapy

Immunosuppressive therapy is the foundation of treatment for LGL leukemia based on the rationale that leukemic LGL represent constitutively activated cytotoxic lymphocytes. First line therapy relies on use of single immunosuppressive oral agents such as methotrexate (MTX, 10mg/m^2/week), cyclophosphamide (100 mg per day), or
cyclosporine (CyA, 3 mg/kg per day). A minimum of 4 months of therapy is required before assessing response. Since the initial publication on the efficacy of MTX, this drug has been considered until now as the best first line option, mainly for neutropenic patients.\textsuperscript{101} Oral cyclophosphamide has been preferentially used in patients with predominant anemia and particularly PRCA.\textsuperscript{60,102} Based on retrospective studies, the overall response rate (ORR) appears quite broad ranging from 21\% to 85\% (median around 50\%), with similar responses to each of the 3 drugs, making comparison difficult. The complete response rate is relatively low, around 21\% for MTX, 33\% for cyclophosphamide, and less than 5\% for CyA.\textsuperscript{9} Duration of response is around 21 months for MTX. However, relapse rate may be high, with the French series reporting rates of 67\%, compared to 13\% for patients receiving cyclophosphamide.\textsuperscript{9,15} Cyclophosphamide as first line therapy may induce high ORR with almost 50\% of CR rate for either neutropenic or anemic patients.\textsuperscript{103} CyA corrects cytopenia without elimination of the LGL clone.\textsuperscript{15,16,104} It has been suggested that HLADR4 (observed in 32\% of LGL leukemia) could be highly predictive of CyA responsiveness.\textsuperscript{105} The results of the first large prospective study of immunosuppressive agents in LGL leukemia was recently reported.\textsuperscript{48} Fifty-five patients received MTX at first line and non-responders were switched to cyclophosphamide. The ORR was 38\% in step 1 (which is lower compared to the results of the large retrospective studies) and 64\% in step 2. These data indicate that a high proportion of patients may respond to cyclophosphamide even after having failed to respond to MTX.\textsuperscript{15,100} As discussed earlier, Stat3 Y640F mutation may be predictive biomarker for response to methotrexate but this observation needs validation in a larger cohort of patients. An important randomized trial (NCT01976182) investigating first line MTX versus cyclophosphamide is ongoing in France will hopefully determine the best choice of initial therapy in this disease.

In case of failure of primary therapy, a switch between MTX and cyclophosphamide should be considered. In our practice, CyA is reserved for patients failing both drugs. Both MTX and CyA are maintained indefinitely as long as these medications are reasonably tolerated and disease response is maintained. Long term use and monitoring of low dose MTX follows guideline recommendations of its use in RA. Hepatic (hepatitis) and lung dysfunction (hypersensitivity pneumonitis) may occur requiring regular evaluation of liver tests and chest X-ray. Our recommendation is to
stop cyclophosphamide after 8-12 months because of its mutagenic potential. Renal function and blood pressure have to be carefully monitored during CyA treatment.\textsuperscript{9} The effects of prednisone as single agent in the treatment of LGL leukemia are not convincing but may decrease some inflammatory symptoms related to RA and in rare cases it may transiently improve neutropenia.

**Second line therapy**

It is difficult to make any treatment recommendations for patients refractory to the first line agents, because of the rarity of the disease and general absence of prospective data. To the authors’ knowledge there is only one ongoing clinical trial in the United States, which investigates alentuzumab.\textsuperscript{110} The US author’s practice is to refer refractory patients for consideration of participation in this study being conducted at the NIH.

Clinical experience using a number of different options in a smaller number of patients has been reported for LGL leukemia patients and these could be considered for refractory patients. These results are summarized below:

i) **Purine analogs.**

Less than 50 patients have been reported to be treated with either fludarabine, 2 CDA, deoxycoformycin, or bendamustine.\textsuperscript{15,104,106,107,108} The overall response rate looks promising at around 79%. Remissions are usually obtained rapidly and the patients may be treated with a maximum of 1 to 3 courses in order to limit toxicity. Fludarabine has been used in combination with Mitoxantrone with long lasting complete remission. Whether or not purine analogs should be integrated into first line therapy is an open question.

ii) **Combined chemotherapy**

Polychemotherapy based on CHOP like or Ara-C containing regimen has not demonstrated efficacy in chronic refractory form of disease and should be reserved for the rare cases of aggressive LGL leukemia. In some cases of multi-refractory patients, stem cell transplantation (SCT) could be considered. We recently reported the EBMT series of 15 patients receiving auto or allogeneic SCT.\textsuperscript{108} Six patients remained disease-free post transplantation.

iii) **Immunotherapy**

Alentuzumab (campath) which is a humanized monoclonal antibody (anti CD52) has been proposed for refractory patients in very limited series. The overall response rate
is around 60%.\textsuperscript{9,15,16,110} However, general availability of this drug is now limited and infectious risks limit use of this agent. Efficacy of extracorporeal photopheresis has been reported recently with documented CR.\textsuperscript{111}

Rituximab, the specific antiCD20 monoclonal antibody, has been paradoxically used in patients having both RA and LGL leukemia. It was suggested that eradication of B cell expansion and autoimmune antigens could have led to eradication of reactive LGL clones. In our opinion, this is not a reasonable option for LGL leukemia.\textsuperscript{112,113}

iv) Splenectomy

Splenectomy may be considered in patients with symptomatic splenomegaly associated or not with cyopenias. An overall review of the literature shows an ORR of 56% but sustained responses are infrequent.\textsuperscript{15,16,114,115}

v) Targeted therapy

Considering the pathogenesis of LGL leukemia, different specific pathways conceivably could be targeted using either cytokine antagonistic agents, monoclonal antibodies (MoAbs) directed against membrane receptors, Jak/Stat inhibitors, NFκB or farnesyl transferase/Ras inhibitors.

Regarding IL15 targeting, a clinical trial (NCT 00076180) has tested effects of Hu-Mikβ1 which blocks IL-15 trans presentation to T cells that express IL-2/IL-15Rβ (CD122). Administration of Hu-Mikβ1 to patients was safe but clinical responses were not observed.\textsuperscript{116} A phase I study with an anti-CD2 monoclonal antibody (siplizumab) was conducted in 2005 in patients with relapsed/ refractory CD2+ T-cell lymphoma/leukemia including T-LGL leukemia (NCT00123942). To our knowledge, the results of this trial have not been published.

Based on constitutive ras activation in leukemic LGL, a phase II study investigating use of farnesyltransferase inhibitor (tipifarnib) enrolled a total of eight patients. No clinical responses were observed despite a significant improvement in marrow hematopoiesis and increased hematopoietic colony growth \textit{in vitro}.\textsuperscript{117}

A Stat inhibitor, OPB-31121, had a significant antitumor effect on leukemia cells with Stat-addictive oncokinases.\textsuperscript{118} Stat3 inhibition could be a good therapeutic option in LGL leukemia. Of interest, low dose MTX was recently identified as a very active inhibitor of Jak/Stat pathway, perhaps now explaining its efficacy in LGL leukemia.\textsuperscript{119} However, in complete responders, STAT3 mutated sub clones are still detected.\textsuperscript{47}

There are recent exciting data concerning Jak3 pathway inhibition. Jak3 is now considered as a target for immunosuppression. Tofacitinib citrate (CP690550), a Jak3
specific inhibitor has demonstrated impressive activity in refractory RA.\textsuperscript{120,121} Recently, 9 patients with refractory LGL leukemia and RA were treated with Tofacitinib citrate. Hematologic response was observed in 6/9 cases, with improvement in neutropenia observed in 5/7 patients.\textsuperscript{122} Since NFκB is constitutively active in T-LGL, Bortezomib could be considered a promising agent for investigation in LGL leukemia as supported by demonstrating efficacy in the LGL leukemia model of IL15 transgenic mice.\textsuperscript{88} In LGL leukemia, proapoptotic signals (ceramide) are downregulated and prosurvival signals (S1PR5) are up-regulated\textsuperscript{85,96}. We showed that FTY720, a S1PR agonist, induced apoptosis of LGL cells and that C6-ceramide encapsulated in nanoliposomes led to apoptosis in an NK-LGL rat leukemia model by targeting survivin. A trial of Ceramide Nanoliposomes is ongoing in patients with advanced solid tumors (NCT02834611) and this could be a potential therapeutic candidate for LGL leukemia.

\textbf{Conclusions}

It appears that immunosuppressive agents such as MTX, cyclophosphamide and CyA are limited in their capacity to eradicate the LGL clone and induce long lasting remission. Due to lack of prospective comparative clinical trials, there is no way to yet decide which one of these three agents is best proposed as first line therapy. There is an urgent unmet need to develop better therapeutics for LGL leukemia, as this disease remains incurable. Very important progress has been made in our understanding of LGL clonal expansion primarily based on the importance of Stat3 activation/mutation in pathogenesis of illness. This knowledge suggests the possibility of using targeted therapy such as Jak/Stat inhibitors, which are currently under development, have entered clinical trials, or already FDA-approved for other medical conditions.
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Drs. Lamy, Moignet and Loughran participated in the writing of this manuscript.

Conflict of Interest Disclosures
Drs. Lamy, Moignet and Loughran declare that there are no conflicts of interest.
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Raposo A, Cerqueira M, Costa J, Sousa Neves J, Teixeira F, Afonso C. Rheumatoid arthritis and associated large granular lymphocytic leukemia-


Table 1: Classification of LGL leukemia according to the WHO classification

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Mature T/NK cell neoplasms</td>
<td>Mature T-cell and NK-cell neoplasms</td>
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<td>Mature T-cell and NK-cell neoplasms</td>
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<tr>
<td>T-cell large granular lymphocyte leukemia</td>
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<td>T-cell large granular lymphocyte leukemia</td>
<td>T-cell granular lymphocytic leukemia</td>
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<tr>
<td>Aggressive NK cell leukemia</td>
<td>Chronic lymphoproliferative disorder of NK cells*</td>
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<td>Aggressive NK-cell leukemia</td>
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<td>Chronic lymphoproliferative disorder of NK cells*</td>
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<td>Modifications:</td>
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<tr>
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<td></td>
<td></td>
<td>- New subtypes recognized with clinicopathological manifestations</td>
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<td>- Stat3 and Stat5b mutations in a subset, latter associated with more clinically aggressive disease</td>
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*Provisional entity
Table 2: Principal associated diseases with LGL leukemia

<table>
<thead>
<tr>
<th>Associated diseases with LGL leukemia</th>
<th>Frequency</th>
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<tbody>
<tr>
<td>Neoplasms</td>
<td>4 to 10%</td>
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<tr>
<td>Autoimmune cytopenia</td>
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<tr>
<td>PRCA</td>
<td>5 %</td>
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<td>AIHA</td>
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<td>ITP</td>
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<tr>
<td>Evans syndrome</td>
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<tr>
<td>B-cell lymphoid neoplasms</td>
<td>5%</td>
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<tr>
<td>Low-grade NHL</td>
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<tr>
<td>DLBCL</td>
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<tr>
<td>Mantle cell lymphoma</td>
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<tr>
<td>Multiple myeloma</td>
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<tr>
<td>CLL</td>
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<td>Hairy cell leukemia</td>
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<tr>
<td>Waldenstrom macroglobulinemia</td>
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<tr>
<td>Hodgkin lymphoma</td>
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<tr>
<td>Lymphomatoid granulomatosis</td>
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<tr>
<td>Heavy chain disease</td>
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<tr>
<td>Autoimmune diseases/connective tissue disorders</td>
<td>10 to 20%</td>
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<tr>
<td>Rheumatoid arthritis</td>
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<tr>
<td>Systemic lupus erythematosus</td>
<td>10 to 18%</td>
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<td>Vasculitis</td>
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<td>Systemic sclerosis</td>
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<td>Endocrinopathy</td>
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<td>APECED</td>
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<td>Type I MEN</td>
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<tr>
<td>Hashimoto</td>
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<td>Grave disease</td>
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<td>CIBD</td>
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<tr>
<td>Celiac disease</td>
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<td>Gougerot-Sjogren syndrome</td>
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<tr>
<td>Glomurolenephritis</td>
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<tr>
<td>Polymyositis</td>
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<tr>
<td>Inclusion body myositis</td>
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<tr>
<td>Poly/multinevritis</td>
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<tr>
<td>RPA</td>
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<tr>
<td>Inflammatory arthritis (unclassified)</td>
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<tr>
<td>Lambert-Eaton myasthenic syndrome</td>
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<td>Good syndrome</td>
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<td>Behcet disease</td>
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<tr>
<td>Multiple sclerosis</td>
<td></td>
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<tr>
<td>Acquired factor VIII inhibitor</td>
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<tr>
<td>Myelodysplasia</td>
<td>3 to 10%</td>
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<tr>
<td>AML</td>
<td>&lt; 1%</td>
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<tr>
<td>Hemophagocytic syndrome</td>
<td>&lt; 1%</td>
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<tr>
<td>Pulmonary hypertension</td>
<td>&lt; 1%</td>
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<tr>
<td>Post organ or hematopoietic stem cell transplant</td>
<td>&lt; 1%</td>
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<tr>
<td>Post viral infection</td>
<td>&lt; 1%</td>
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</tbody>
</table>

*Some patients presented with more than one auto-immune associated disease
Figure 1: Large granular lymphocytes on blood (A) and marrow smears (B)

Fig 1A: A typical large granular lymphocyte on blood smear. (Wright-Giemsa stain: original magnification x 1000 Caméra RETIGA 2000). (with the courtesy of Dr Ly-Sunnaram. Rennes University Hospital)

Fig 1 Bone marrow smear showing numerous LGL with a basophilic neutrophil. (Wright-Giemsa stain: original magnification x 1000 Caméra RETIGA 2000). (with the courtesy of Dr Ly-Sunnaram. Rennes University Hospital)
Fig 2: Flow cytometry and TCR γ gene rearrangement analysis in LGL leukemia


B: Flow cytometry analysis of a case of NK CD3- LGL leukemia showing a CD3-/CD8-/CD16+/CD56+ phenotype (pink). KIR monotypic expression using CD161 and NkB1.
C: Clonality assessment of the same case of fig 3A: (Left: specific Vβ MoAbs showing restricted expression of Vβ13.1 > 90%, Right: detection of clonal TCR γ gene recombination by geneScanning analysis, single peak in green).
D: Stat 3 mutation detection in a case of T LGL leukemia using Sanger sequencing.
Figure 3: Algorithm of the diagnosis of LGL Leukemia

Clinical or biological context: neutropenia, recurrent infections, anemia, autoimmune conditions, rheumatoid arthritis, lymphocytosis

Careful blood smears examination: excess of large granular lymphocytes?

Flow cytometry analysis using CD3/5/4/8/16/56/57, TCRγ rearrangement, (Stat 3 mutation: recommended at this stage, Vβ repertoire and KIR phenotyping; if available.)

Polycional
- Reactive LGL proliferation
- Post-splenectomy, viral infection (CMV, HIV, EBV)
- Bone marrow/organ transplantation,…

Grey Zone
- Low LGL count around 0.5-1 x 10.9/L, and no blood clonal evidence, stat3 unmuted, pancytopenia, lymphopenia, with clinical evocative context
- Bone marrow biopsy with Immunohistochemistry

Interstitial and intravascular subtle LGL
- Infiltration of clusters of CD3+/CD8+ Granzyme B/TIA1+ LGL
- Lymphoid nodules of B and T CD4 cells

True clonal LGL expansion
- T or NK LGL Leukemia
- Clonal TCRγ

Additional tests (if required and not done before)
- Stat3-5b / NFKB, mutation
- Vβ repertoire, KIR phenotyping
Figure 4: Bone marrow features of LGL leukemia

H&E staining of marrow biopsy reveals a slightly hypercellular marrow with subtle increase in interstitial lymphocytes (A). CD3 staining reveals the LGL interstitial infiltration. (B). Immunoperoxidase staining for CD8 demonstrates clusters of at least 8 cytotoxic lymphocytes CD8+. (C) Linear array of intravascular LGL demonstrated by Granzyme B staining (D). CD57 staining reveals the LGL interstitial infiltration (E). (with the courtesy of Pr Gaulard and Dr Tas)
Figure 5: model of LGL Leukemia Pathogenesis

Figure Legends: Activation and expansion occur, resulting in an oligoclonal cytotoxic lymphocyte population (colored outline represents a distinct clone, blue triangle represents an unknown antigen). STAT3 is activated and may acquire a mutation. Chronic antigen stimulation leads to expansion of one dominant (monoclonal) cytotoxic lymphocyte population (all are outlined in black signifying the monoclonal population). Three outcomes occur. In panel 1, production of inflammatory cytokines (starburst shapes representing: IFN-γ, IL-8, IL-10, IL1-β, IL-12p35, IL-18, IL1Ra, RANTES, MIP1-α, MIP1-β) causes symptoms such as neutropenia, anemia, and fatigue, and can also cause autoimmune disease such as rheumatoid arthritis (RA). In panel 2, the functional killer cells release cytotoxic granules containing perforin and granzyme B (purple dots); this leads to the same outcomes as in 1. In panel 3, the STAT3-mediated survival network results in a persisting clone due to profound dysregulation of apoptosis, including resistance to Fas/FasL mediated death and upregulation of Mcl-1.
Figure 6: Key molecular abnormalities in LGL leukemia

**Figures Legends:**

**Fas-mediated DISC inhibition:** LGL leukemia cells are resistant to Fas mediated apoptosis. Soluble Fas (sFas) acts as a decoy receptor able to inhibit Fas-dependent apoptosis. Increased level of an inhibitory protein named c-FLIP contributes to the DISC formation defect. 

**Jak/Stat3 pathway:** Stat3 is constitutively activated in LGL leukemia and is responsible for the transcription of Bcl2 and Mcl1 protein expression. Inhibition of Stat3 restores apoptosis of LGL cells whatever Stat3 mutation status which implies that Stat3 mutation is not itself mandatory to explain LGL clonal expansion. SOCS3 which inhibits Jak/Stat3 pathway is significantly decreased in LGL leukemia. 

**Survival signal:** LGL leukemia shows a predominant expression of pro-survival sphingolipids (S1P). SphK1 which converts sphingosine into SP1 is increased in LGL leukemia and SphK1 inhibition leads to leukemic LGLs apoptosis. SP1 binding to SP1R activate pro survival signal through ERK1/2 signaling. Moreover, expression of S1P receptor, mainly S1PR5, is increased in LGL leukemia. Ras-Raf-1-MEK1-ERK, PI3K/AKT pathway are upregulated in LGL leukemia and they inhibition lead to LGL apoptosis. Increased activity of the PI3K-AKT signaling axis is found in T-LGL cells and participate to apoptotic inhibition. NFKB activity is upregulated in LGL leukemia. NFKB acts downstream of the PI3K-AKT pathway to prevent apoptosis through Mcl-1 independently of Stat3. A recurrent non-synonymous mutation in the gene encoding an NFKB signaling inhibitor, TNFAIP3, was found in 8% of LGL leukemia patients.

**IL15 and PDGF:** IL15 promotes myc expression through NFKB pathway (model of IL15 transgenic mouse). IL15 is associated to an increase of global DNA methylation level in LGL leukemia through DNMT3A upregulation. The down regulation of miR-29 is responsible for the up regulation of DNMT3A which induce methylation of the tumor suppressor gene Ibd4.