15 Large Granular Lymphocyte Leukemia

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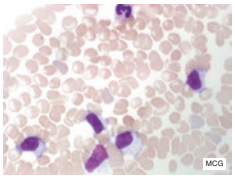
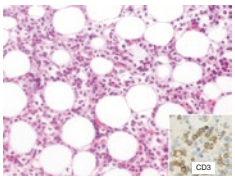
T-cell large granular lymphocyte leukemia (T-LGL)

Clinical outline
Adults with large granulated lymphocytes in peripheral blood, bone marrow and spleen and liver. Usually no lymphadenopathy. Severe neutropenia and anemia. Frequent associated with autoimmune conditions, such as rheumatoid arthritis or hematological malignancies. May arise in the post-transplant setting.

Cytology	Medium to large lymphoid cells with condensed chromatin and abundant cytoplasm with azurophilic granules.	T-large granular lymphocytes leukemia, cytology 
Histology	Sinusoidal and interstitial pattern in bone marrow. Associated reactive lymphoid aggregates. Left-shifted and/or reduced hematopoiesis. In spleen expanding the cords and the sinusoids of the red pulp, sparing the whit pulp.	T-large granular lymphocytes leukemia, hystology 

	CD20	CD3	CD5[1]	CD4[2]	CD8[2]	CD7[1]	CD56[3]	TCR[4]	CD57	CD30
notes	[1]weak/dim, [2]small subset is CD4+ and CD8-, [3]small subset CD56+ and CD57-, [4]Tcell receptor usually αβ, rarely γδ- phenotype									
other marker	Consistent with its activated cytotoxic nature, expression of TIA-1, perforin, granzyme-B and granzyme-M is commonly observed.									

= majority of cases positive = variable fraction of cases positive = negative

Main differential diagnosis	Reactive T-cell expansion (should be polyclonal); chronic lymphoproliferative disorder of NK cells (should be CD3-, CD57-, CD56+; no TCR rearrangement); hepatosplenic T-cell lymphoma (should be CD56+, majority TCRγ+, TCRαβ- (with some exceptions expressing TCRαβ) CD4-, CD8-/+, CD57-, granzyme B-, perforin-).

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Key molecular features
Clonally rearranged T-cell receptor (TCR) genes, restricted TCR repertoire.
Frequent translocations: not reported.
Frequent copy number alterations: not reported.
Frequent mutations: *STAT3*, less common *STAT5b*
Precursor lesions
Not known.
Progression
Only anecdotic reports of transformation to other T-cell lymphoma

Clinically relevant pathologic features	**Relevance**	**Evidence**
***STAT3* mutation**	Predictive: better response to first line, immunosuppressive therapy; may be suitable for target therapy	**B**
***STAT5b* mutation**	Prognostic: identifies cases with more aggressive course	**C**
Legend: A= verified in multiple studies, randomized trials and/or integrated in guidelines; B= variable between studies/ needs definitive validation; C= preliminary / discrepant results.		

15.1 Introduction

The 2017 WHO classification reports large granular lymphocyte (LGL) leukemia in the category of cytotoxic T and NK cell lymphoma and leukemia. LGL leukemia is a lymphoproliferative disorder, defined by the presence of a high percentage of circulating LGLs in peripheral blood. The lymphocytosis is chronic and is sustained by clonal mature T or NK cells, thus configuring T-LGL leukemia (T-LGLL) or chronic lymphoproliferative disease of NK cells (CLPD-NK), respectively. T-LGLL is the most frequent form, accounting for about 85% of cases, whereas NK form is less represented with 10% of cases. In this scenario, also rare cases (incidence 5%) of aggressive LGL disorders of T or NK lineage are included, with very poor prognosis. Among these latter, the T-related type is described in the literature, but it is not yet included in the WHO classification that actually recognizes only the NK form, referred to as aggressive NK cell lymphoma (ANKL), more frequently found in oriental populations and usually associated with Epstein-Barr virus (EBV) infection. The etiopathogenesis of LGL leukemia has not been established, but it is hypothesized that a viral or autologous antigen triggers the initial lymphocytosis whose survival over the time is then maintained by the activation of many cell pathways. Among these, JAK/STAT3 axis is claimed as the principal signal involved in cell imbalance toward cell survival, the signal transducer and activator of transcription 3 (STAT3) activation being the hallmark of LGL disease. Moreover, in about 40% of patients, mutations on *STAT3* have been recognized that further sustain the activation of this pathway.

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T and NK cell disorders share several biological and clinical features. Both entities are characterized by STAT3 activation, and both types of patients show clinical manifestations due to cytopenia. Furthermore, the treatment of T-LGLL and CLPD-NK patients is very similar, entailing an immunosuppressive regimen or careful observation of chronic lymphocytosis.

15.1 LGL Leukemia: Clinical Aspects

15.1.1 Leukemic LGL Cytology and Immunophenotype

On blood films, the LGL nucleus is typically round with condensed, mature chromatin; the cytoplasm is pale and abundant with randomly distributed azurophilic granules containing perforins and granzyme conferring its cell lytic ability [1] (Fig. 15.1). Leukemic LGLs do not show morphologic difference with LGLs in healthy individuals.

The immunophenotype is central to identify LGLs among lymphocytes. The presence/absence of CD3 expression on cell surface distinguishes LGL belonging to T or NK lineage, respectively.

CD3+ LGL leukemia cells usually express the TCR αβ+, CD4−, CD8+ phenotype, and the disease may be also referred to as CD8+ T-LGLL. Less frequently, in 10–15% of cases, the disorder is sustained by TCR αβ+, CD4+, CD8+/− LGLs, often expressing TCR Vβ13.1 [2], defining the CD4+ T-LGLL. T-LGLs are also typically equipped with CD16, CD56, and CD57. CD57 is almost always present, and it is considered a specific marker of LGL, whereas CD16 and CD56 may be present or absent in different combinations. The rare cases with T-LGLs CD57 negative are usually characterized by the expression of CD16. In T-LGLL, CD5 and CD7 are weakly expressed, and LGLs usually display a cytotoxic phenotype corresponding to that of a fully differentiated mature cytotoxic T lymphocyte (CD45RA+, CD27−, CD28−, CD62L−, CCR7−). Leukemic LGLs are constitutively equipped with IL-2Rβ (CD122), but not IL-2Rα (CD25), and can variably express NK receptors as CD94/NKG2 (A or C) and killer immunoglobulin-like receptors (KIRs) [3].

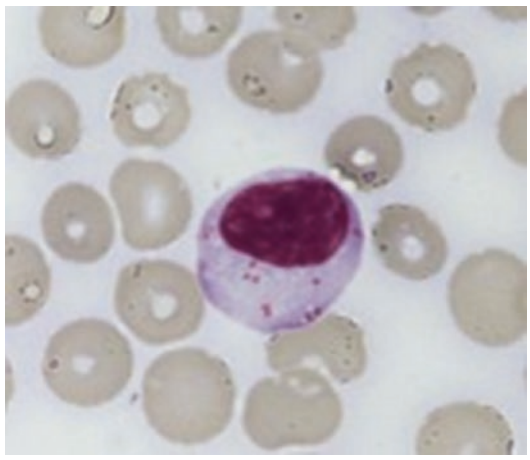

Fig. 15.1 May-Grünwald-Giemsa staining of a large granular lymphocyte in peripheral blood

Beyond the expansions of T cells bearing the TCR αβ+, there is a minority of cases derived from TCRγδ+ cells, presenting a Vγ9+/Vδ2+ or, less frequently, a Vγ9−/Vδ1+ phenotypic profile with TCRγ monoclonal restriction [4].

Some cases of a rare aggressive form of T-LGLL were reported, and these cases are usually equipped with a phenotype CD3+, CD8+, and CD56+ but devoid of CD16 and CD57 expression [5].

CLPD-NK are characterized by the lack of TCR and the CD16+, CD56+, CD45RA+, CD122+, CD25− phenotype. CD57 antigen is usually weakly detectable. CD56 antigen is normally expressed by LGL, although some negative cases are reported. CD94 antigen is found at high density on patients' NK cells; this antigen is usually associated with the inhibitory subunit NKG2A, although in some cases the association CD94/NKG2C has been reported [6]. Patients' NK cells characteristically express functional β and γ chains of IL-2/IL-15 receptor, which are strictly related to the role of these cytokines in the pathogenesis of disease [7]. Expression of NK receptors, mostly represented by KIR, is altered in patients with CLPD-NK. A restricted pattern of KIR expression is currently used in

these patients to define the clonal nature of NK cell proliferation [6, 8, 9].

The aggressive and rare ANKL is not provided by an immunophenotype specifically distinguishable from CLPD-NK, and clinical evaluation is necessary to make the diagnosis. The presence of large nucleoli in NK cells strongly supports the diagnosis of ANKL, associated with age <40 years, systemic symptoms, lymph node swelling, and hepatosplenomegaly [10, 11].

Some representative cytometer panels of the most frequent immunophenotype for each LGLL subtype are reported in Fig. 15.2.

15.1.2 Diagnosis

Historically, the evidence of a lymphocytosis greater than 2 x 10^9 LGL/L, lasting for more than 6 months and clonal, was requested for the diag-

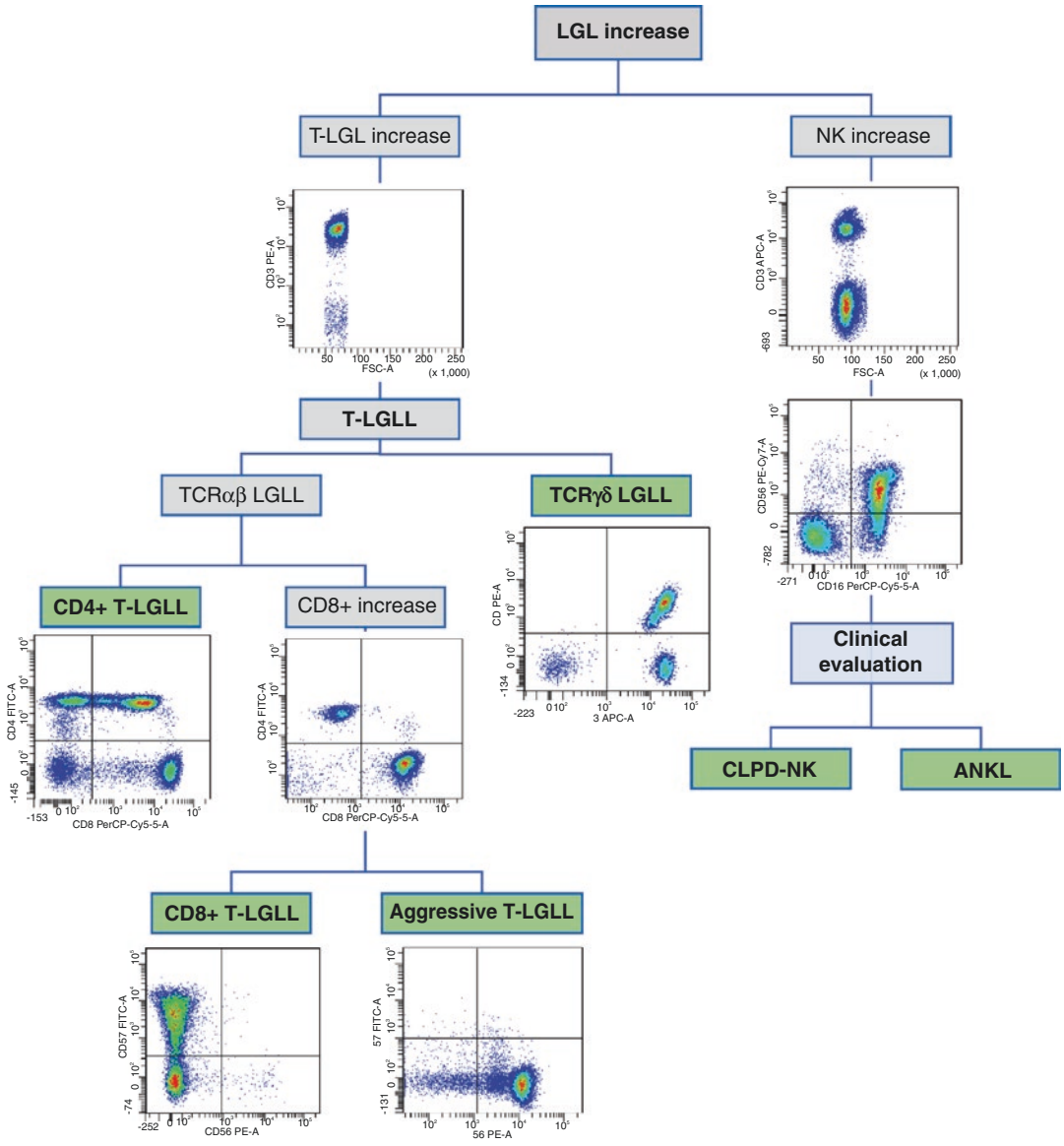

Fig. 15.2 Algorithm of the flow cytometer evaluation of the immunophenotypic features on LGL leading to the different types of LGL disorders

nosis of disease [1], considering that normally circulating LGL count is 0.25 × 10^9/L. However, the demonstration of clonal restriction of LGL proliferation can often be provided even in the presence of less than 2 x 10^9 LGL/L, particularly in symptomatic disease. For this reason, the threshold actually accepted for diagnosis is 0.5 x 10^9 LGL/L [12]. Additionally, the lymphocytosis must be characterized by immunophenotyping and molecular analyses.

As detailed in the previous paragraph, a proper definition of LGL immunophenotype is mandatory to recognize LGL lymphoproliferation and to define LGL leukemia subtypes: Tαβ LGLL including CD8+ T-LGLL, CD4+ T-LGLL, and aggressive T-LGLL; Tγδ LGLL; and CLPD-NK.

The molecular analysis is critical to distinguish reactive LGL proliferation from true LGL leukemic clonal expansion. Polyclonal expansions of LGLs are usually transient and due to a viral infection, such as EBV or cytomegalovirus (CMV), neoplasms, or autoimmune diseases [13–16]. Sometimes, this condition may develop after splenectomy. In contrast, clonal LGL proliferations are stably maintained for time, whether or not the patients are symptomatic.

The T-LGL leukemia can be tested for clonality by TCR rearrangement study. TCR gamma polymerase chain reaction analysis is a routine and easy technique to assess T-LGL clonality in both Tαβ and Tγδ LGLL. Showing the preferential use of one or two TCR-Vβ segments, flow cytometry analysis with monoclonal antibodies against the various Vβ regions of the TCR allows to suggest, and then used as a surrogate marker, the clonality of the LGLs [17]. The current Vβ monoclonal antibody panel covers 75% of the Vβ spectrum, with a high correlation between Vβ flow cytometry and TCRγ-polymerase chain reaction results. No prevalence of any of Vβ was reported in T-LGLL but Vβ13.1 in CD4+ T-LGLL [2]. These techniques should be applied to all suspected cases and are useful in those patients in whom the absolute LGL count is not significantly increased. To these routinely used investigations, deep sequencing (NGS) of TCR has recently emerged to be a useful tool to demonstrate restricted diversity of TCR repertoire [18], not only supplying information about the clonal nature of LGL expansion but also providing the number and the entity of clones included in LGL increase and the identification of the CDR3 sequence. NGS analysis has been proven to be also useful to monitor therapy response and minimal residual disease.

The clonality of CLPD-NK is more difficult to assess, NK-LGLs being not equipped with the TCR. In this case, chromosomal abnormalities or the restriction fragment polymorphism of X-linked gene analysis can provide the proof of clonality. A KIR-restricted expression pattern, demonstrated by flow cytometry analysis, has been accepted as a surrogate marker of monoclonal expansion [8].

At the diagnosis, when the criteria are difficult to be satisfied, histologic bone marrow (BM) immunochemistry analysis is requested. LGLs in BM appear as individual cells or small clusters localized in sinusoids. Since the amount of infiltrating cells is usually low, BM involvement by leukemic LGLs is often difficult to identify, even when LGL expansion is apparent in peripheral blood [19, 20].

15.1.3 Clinical Features

LGL leukemia is an extremely rare disease representing 2–5% of chronic lymphoproliferative disorders in North America and Europe and 5–6% in Asia. Two registries, one Dutch and one American, report the incidence of LGL leukemia as 0.72 and 0.2 cases per one million of individuals per year, respectively [21, 22]. T-LGL leukemia patients have reduced survival compared with general population, with a median overall survival of 9 years [12].

The disease usually affects old people (mean 60 years), men or women with the same proportion. The disease runs asymptomatic in nearly 40% of cases, with lymphocytosis representing the only observed hematological abnormality [23, 24]. The definition of T-cell clonopathy of unknown significance (TCUS) has also been suggested to designate these asymptomatic patients [25]. Disease may run asymptomatic for many

years; however, during the course of disease in 60% of cases, therapy is needed, mostly for cytopenia-related manifestations. Symptomatic patients show clinical complications more frequently due to neutropenia, as fever caused by infections or mouth lesions. Recurrent infections are reported in 15–39% of cases [26]. Neutropenia is defined as an absolute neutrophil count (ANC) less than 1.5×10^9/L and severe neutropenia with ANC $<0.5 \times 10^9$/L. However, some patients with severe and persistent neutropenia can be also devoid of infections for a long period of time; in these cases, therapy can be delayed. Weakness due to anemia represents another relevant finding, with 10–30% of cases being transfusion-dependent. B-related symptoms (fever, night sweats, weight loss) are observed in nearly 25% of cases. Thrombocytopenia is generally moderate and is found in less than 25% of cases, whereas pure red cell aplasia (PRCA) occurs in 8–19% of the cases [27].

Table 15.1 Conditions associated with the lymphoproliferative disease of granular lymphocytes

Associated disease	Frequency
Autoimmune disease	15–40%
Rheumatoid arthritis	25%
Systemic lupus erythematosus	<5%
Still disease	<5%
Systemic sclerosis	<5%
Vasculitis	<5%
Polymyositis	<5%
Poly/multineuritis	<5%
Autoimmune cytopenia	5–10%
Pure red cell aplasia	5%
Autoimmune hemolytic anemia	<5%
Idiopathic thrombocytopenic purpura	<5%
Hematological malignancies	<10%
MGUS/multiple myeloma	<5%
Myelodysplasia	<5%
Myelofibrosis	<5%
Hodgkin's and non-Hodgkin's lymphomas	<5%
Pulmonary arterial hypertension	<5%
Chronic viral infection (EBV, HTLV, CMV, HIV, HCV)	<5%

MGUS monoclonal gammopathy of undetermined significance, *EBV* Epstein-Barr virus, *HTLV* human T-lymphotropic virus, *CMV* cytomegalovirus, *HIV* human immunodeficiency virus, *HCV* hepatitis C virus

Frequently, patients may have other associated conditions; among these, rheumatoid arthritis (RA) is the most commonly reported comorbidity condition [12, 28], but several other connective tissue diseases, including systemic lupus erythematosus, vasculitis, and polymyositis, have been reported [29–32]. Hematological disorders are another well-represented group including monoclonal gammopathies, multiple myeloma, myelodysplastic syndromes, myelofibrosis, and Hodgkin's and non-Hodgkin's lymphomas [12, 28]. An association has been established between LGL disorders and pulmonary hypertension, with documented infiltration of the lung by LGLs, and inclusion body myositis has occasionally been reported [32]. From 20% up to a half of the patients have splenomegaly, around 20% of cases present skin lesions, and only a minority have hepatomegaly; lymphadenopathy is rare [33]. Diseases associated with LGL leukemia are listed in Table 15.1.

15.1.4 The Predicting Value of the Immunophenotype

Intriguingly, a correlation between immunophenotype and clinical and genetic features has been reported [34, 35], even if the nature of this relationship needs to be elucidated. In CD8+ T-LGLL, it has been demonstrated that CD16+/CD56− phenotype, with or without CD57, is strongly linked to patients characterized by neutropenia and the presence of *STAT3* mutation [35]. The association between *STAT3* mutation and symptomatic disease has been further reported in several papers [36–38]. The rare aggressive form of T-LGLL is characterized by proliferations of CD8+/CD56+/CD16−/CD57− LGLs, and patients are frequently mutated in *STAT5b* [39]. Interestingly, CD4+ T-LGLL patients are always CD8±/CD56+ and include patients carrying *STAT5b* mutations, but they never show to carry *STAT3* mutations, and they are almost always characterized by an indolent clinical course [35, 40].

In 2017 WHO classification, *STAT3* and *STAT5b* mutations are introduced to be consid-

ered for the identification of a subset of patients, *STAT5b* mutations being associated with a more aggressive disease. Anyway, this consideration will need to be updated with the discovery of *STAT5b* genetic lesions also in indolent CD4+ T-LGLL [40].

Similar to T-LGLL, discrete subtypes of CLPD-NK can be identified by flow analysis depending on the intensity of CD56 and CD16 expression and on CD57 presence or absence. Interestingly, patients characterized by $CD56^{-/dim}/CD16^{high}/CD57^{-}$ cytotoxic NK cell expansion include a unique phenotypic subgroup characterized by a more symptomatic disease and the presence of *STAT3* mutation [41].

The dominant LGL immunophenotypes indicative for clinical presentation are schematically reported in Fig. 15.3.

15.1.5 Therapy

The percentage of patients requiring therapy during the natural history of the disease ranges from 30% to 70%, according to different series [12, 28]. Indications for treatment include severe and symptomatic neutropenia (associated with recurrent infections), transfusion-dependent anemia, or thrombocytopenia as far as progressive disease (i.e., appearance of organomegaly, B symptoms, and rapidly LGL raising counts). Correction of cytopenias with therapy may be achieved without eradication of the clone, which often persists even after treatment. Given that LGLs are activated cytotoxic lymphocytes, therapy is based on immunomodulatory drugs. Methotrexate (MTX, 10 mg/m^2/weekly), low-dose cyclophosphamide (CTX, 50–100 mg/day), or cyclosporine A (CyA, 3–5 mg/kg/day) are commonly used [42, 43]. Corticosteroids may be useful as part of the initial treatment to accelerate response, and growth factors are often used as supportive care. Splenectomy may be considered as an adjuvant in patients with relevant splenomegaly and refractory cytopenias [44].

The first-line therapy relies on the use of single immunosuppressive oral agent, and up to now MTX or CTX have been considered as the best first-line choice. Moreover, MTX is reported to be more efficient for neutropenic and *STAT3* Y640F mutated patients [42, 45].

Once patients with LGL leukemia start treatment, the regimen should be continued for a period of at least 4 months, and they should be closely observed through complete blood counts [43]. After this time point, a hematological complete response is considered achieved when blood counts reveal platelets >150 × 10^9/L, ANC >1.5 × 10^9/L, lymphocytes <4 × 10^9/L, and hemoglobin >12 g/dL. Complete molecular remission is reached when the T-cell clone is no longer detectable through PCR analysis [43]. Partial response is considered when overall blood counts improve, but the ANC has not achieved levels

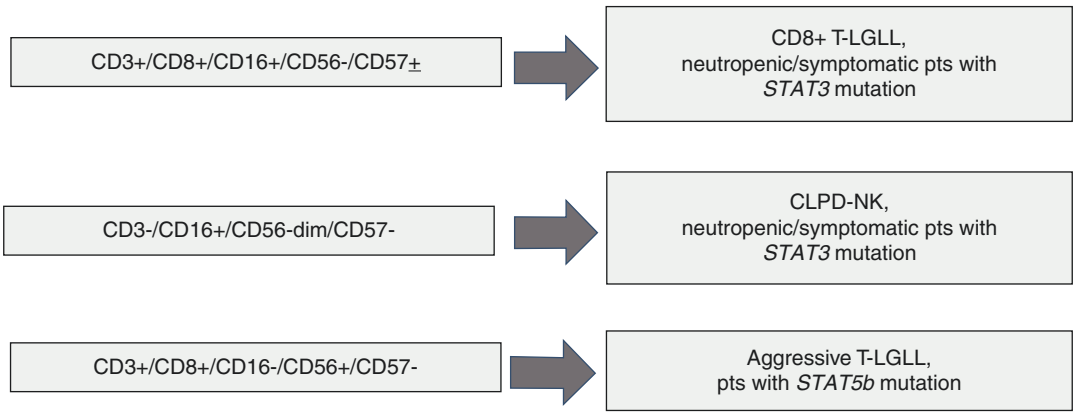

Fig. 15.3 The LGL immunophenotypes indicative for clinical and biological presentations

> 0.5 x 10^9 cells/L, still leaving the patient at potential risk for secondary infections. If an improvement is not achieved after 4 months of continued treatment, one of the alternative therapies described above must be taken into account. However, evidence is accumulating for continuing treatment once established for a longer period of time (usually 1 year), before changing therapy.

The overall response rate (ORR) ranges from 21% to 85%, with similar responses to each of the three drugs. The complete response (CR) rate is 21% for MTX, 33% for CTX, and 5% for CyA [43]. Unfortunately, when the clinical response occurs, patients frequently relapse, and new therapeutic strategies are needed.

Chemotherapeutic agents, such as gemcitabine, liposomal doxorubicin, and bendamustine, and the purine analogs, such as fludarabine, cladribine, and nelarabine, represent possible new agents to be considered for symptomatic LGL disorders [26, 28]. These molecules have been reported to be promising but only in few patients. The use of these agents should be considered for young patients as they allow the achievement of good remissions, including the reduction of bone marrow infiltration.

In some patients with refractory disease, stem cell therapy may be considered. In a series of 15 patients receiving auto- or allogeneic stem cell therapy for LGL leukemia, six patients remained disease-free after transplantation [46].

Monoclonal antibodies anti-CD52 (Campath-1H) and anti-CD122 have been incorporated in the therapeutic scenario for refractory patients. However, the use of anti-CD52 is restricted for its limited availability and infection risks, and the administration of anti-CD122 to patients did not show any response [26, 47]. Similarly, also a phase 2 study with the use of RAS farnesyltransferase inhibitor tipifarnib, according to the finding of a constitutively active signaling of Ras/MAPK/ERK pathway, has unfortunately led to unsatisfactory clinical response [48]. Rather, JAK3-specific inhibitor (tofacitinib citrate), tested in refractory patients, and the multicytokine inhibitor BNZ-1, tested in phase 1 trial, showed promising responses [49]. In addition, the proteasome inhibitor bortezomib has been reported to display anticancer activity against aggressive NK leukemia and extranodal NK/T cell lymphoma *in vitro* and *in vivo*, opening new therapeutic perspectives for LGL patients [50, 51]. Recent data support this approach [52].

Treatment of the rare forms of aggressive LGL leukemia includes polychemotherapy based on CHOP-like (CTX, doxorubicin, vincristine, and prednisone) or cytosine arabinoside-containing regimens [26], usually with unsatisfactory results.

15.2 LGL Leukemia: Molecular Aspects

15.2.1 Pathogenesis

The etiology of LGL leukemia remains still unknown, but some crucial cornerstones for disease development have been identified. It is supposed that no single, specific agent can finally trigger the LGL proliferation. In fact, the proliferation and accumulation of a transformed T or NK cell might represent the expression of a dysregulation of cytotoxic LGL homeostasis because of persistent antigenic drive in combination with immunogenetic factors favoring persistent cell expansions [53]. Moreover, the recognized role in LGL survival, played by some inflammatory cytokines and monocytes, dendritic cells, and mesenchymal stromal cells (MSC), supports the involvement of an inflammatory environment in the pathogenesis of the disease (Fig. 15.4).

15.2.2 The Inciting Event

Many reports strongly support the role of a chronic/persistent antigenic stimulation by auto-antigens or foreign infective antigens as the initial step. This would lead to the expansion of a fully differentiated effector cytotoxic LGL which is not cleared as a consequence of an impairment of apoptotic pathways [53, 54].

Supporting the involvement of auto-antigens, dysregulated autoimmune responses are fre-

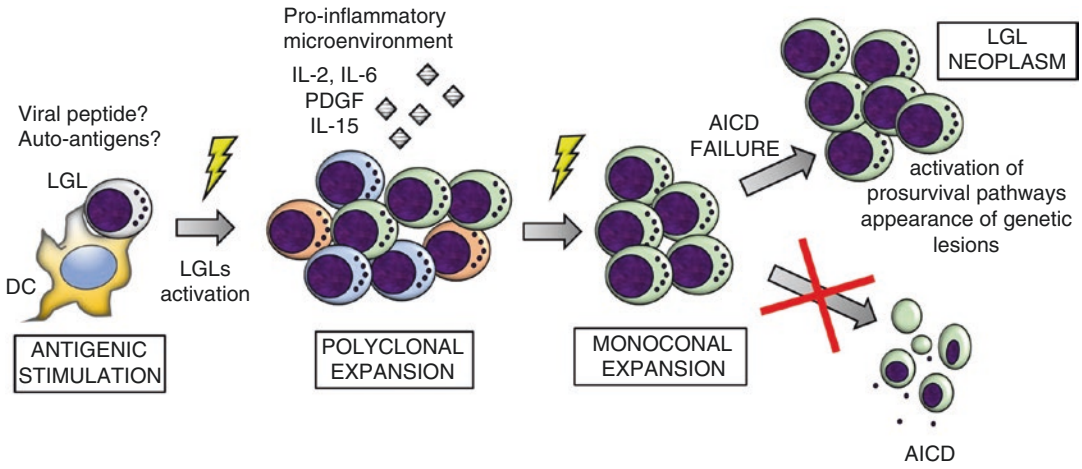

Fig. 15.4 Schematic representation of the pathogenetic hypothesis of large granular lymphocytes leukemia. *LGL* large granular lymphocyte, *DC* dendritic cell, *AICD* activation-induced cell death

quently demonstrated in LGLL patients [15, 55], such as the presence of rheumatoid factors and antinuclear antibodies, rheumatic diseases being commonly associated with LGL leukemia (Table 15.1).

Several reports support a possible role of viral antigens. The pathogenic role of EBV or human T-cell leukemia viruses (HTVL) in some cases of LGL disorder has been reported [14, 16, 56]. Although no prototypic HTLV infection was demonstrated, the evidence that sera from a series of cases from Europe and USA react with the recombinant HTLV env protein p21E, specifically in BA21 epitope, indicates that exposure to a protein containing homology to BA21 may be important in the pathogenesis of this lymphoproliferative disorder [57]. Similarly, evidence has been provided that chronic stimulation of T cells by CMV leads to a persistent clonal expansion of CD4+/CD8−/+dim LGLs, with a predominance of TCR Vb13.1 usage in individuals with an HLADRB1*0701 haplotype [58]. The hypothesis has been formulated that a persistent CMV stimulation can trigger and maintain the LGL clone in patients with a genetic predisposition.

All these data show that not an exclusive and unique factor may be responsible for the initial event causing LGL expansion but rather that multiple factors, referred to auto/viral antigens, may be alternatively involved.

15.2.3 Bone Marrow Involvement

As stated before, LGLL patients usually present leukemic infiltration in bone marrow, where clonal LGLs cluster in small lymphoid aggregates or in microvascular structures [19]. LGLL patients' bone marrow was also demonstrated to be fibrotic for the induction of high collagen (types I, III, and V) deposition by MSC, finally leading to an impaired hematopoietic stem cell proliferation. Together with LGL infiltration, fibrosis has been reported to correlate with the presence of cytopenia [59].

It has been proposed that bone marrow represents the setting in which the putative antigen presentation takes place. Furthermore, dendritic cells (DCs) have been suggested to represent the target of infection in these patients [60]. In fact, a co-localization of DCs and leukemic LGLs has been identified both in T-LGLL and in CLPD-NK. Moreover, bone marrow-derived DCs induced a strong proliferation of autologous purified LGLs from T-LGLL patients pointing that DCs pulsed with a specific antigen might be the putative-inciting agent responsible for T-LGL proliferation [60]. On the contrary, the clonal expansion of NK cells might be due to an impairment in NK/DC equilibrium in the bone marrow, because leukemic NK cells failed to induce DC maturation due to a NKp30 down-modulation [61].

15.2.4 Peripheral Blood Inflammatory Cytokines

Several proinflammatory cytokines have been identified to be higher in LGLL patients' plasma in comparison to healthy controls, such as IL-1β, IL-1Rα, IFNγ, CCL5, CCL4, IL-18, IL-8, CXCL10, CXCL9, and IL-6. Most of them can be related to immune cytotoxic response after viral infection or to RA, which is often associated with LGLL [62–66]. The role of IL-6 was demonstrated: IL-6 and its specific receptor, IL-6Rα, were found to be higher in patients' plasma, and they were released by the non-leukemic fraction of the mononuclear cells; IL-6 contributed to the survival by stimulating STAT3, a key protein in LGLL development [66].

IL-15 is another cytokine that was proven to be important in LGLL pathogenesis both *in vitro* and *in vivo*. IL-15 was shown to induce LGL cytotoxicity and proliferation through proteasomal degradation of the pro-apoptotic protein Bid [7, 67]. The pathogenetic role was demonstrated by the generation of IL-15 transgenic mice (IL-15tg) as they developed a fatal clonal NK and memory CD8+ expansion [68]. IL-15 induced chromosomal instability and DNA hypermethylation via repression of *mir*-29b and the induction of a Myc/NF-kB/DNMT3a axis [51]. Nevertheless, the lethal expansion was induced only when there was a *cis* activation by IL15Rα. In fact, the specific receptor IL-15Rα was expressed by LGLs, and it was detectable in high amounts in patients' plasma [7, 69]. Further supporting the role of IL-15 in the pathogenesis, a systematic biology approach identified IL-15 and PDGF as master survival signaling switches that may have a profound effect on all known deregulations in T-LGL leukemia [70].

15.2.5 Clonal Drift

The phenomenon of clonal drift supports the theory that the emergence of LGL clone might be caused by the recognition of different epitopes of the same chronic antigen. This phenomenon is characterized by the change of the dominant LGL clone over the time. It has been reported that 37% of T-LGLL patients displayed a change in the dominant clone, developing a different Vβ clone instead of the original one owned at diagnosis as demonstrated by Vβ typing [71]. In another series of 42 patients with KIR-restricted CLPD-NK, the presence of monoclonal T cell populations in 48% of cases was also demonstrated. These T monoclonal populations can be detected at the time of diagnosis or occur during the natural history of disease, indicating that the association of T and NK proliferations is more frequent than initially thought. The T cell clone can eventually become so relevant to be dominant, leading to the shift from CLPD-NK to T-LGLL [72]. Similarly, also a T-LGLL patient who developed CLPD-NK over time has been described [73] (Fig. 15.5). All these observations indicate that cells are under antigenic pressure, suggesting that the putative antigen is likely to persist for many years and possibly for the lifelong of patients.

15.2.6 AICD Failure

Physiologically, during infection exposure or antigen stimulation, LGLs undergo proliferation and, after antigen clearance, are eliminated by a process called activation-induced cell death (AICD). This process leads to cell death through the induction of a death-inducing signaling complex (DISC). In LGLL patients, LGLs do not undergo apoptosis for a dysfunctional AICD mechanism, leading to the increase of leukemic cells in the peripheral blood. In detail, LGLs are not sensitive to Fas-induced apoptosis [74], a process essential for AICD [75], and possess high levels of c-FLIP, a DISC inhibitory protein [53].

15.2.7 JAK/STAT Pathway

In addition to the deregulation of apoptosis mechanism, in leukemic LGLs, multiple cell survival pathways have been found to be constitutively activated. Among these, JAK/STAT3 is the main involved axis. In fact, the activated (phosphorylated) form of STAT3 characterizes all leukemic LGLs, and it is currently considered the disease hallmark. The finding that the JAK-

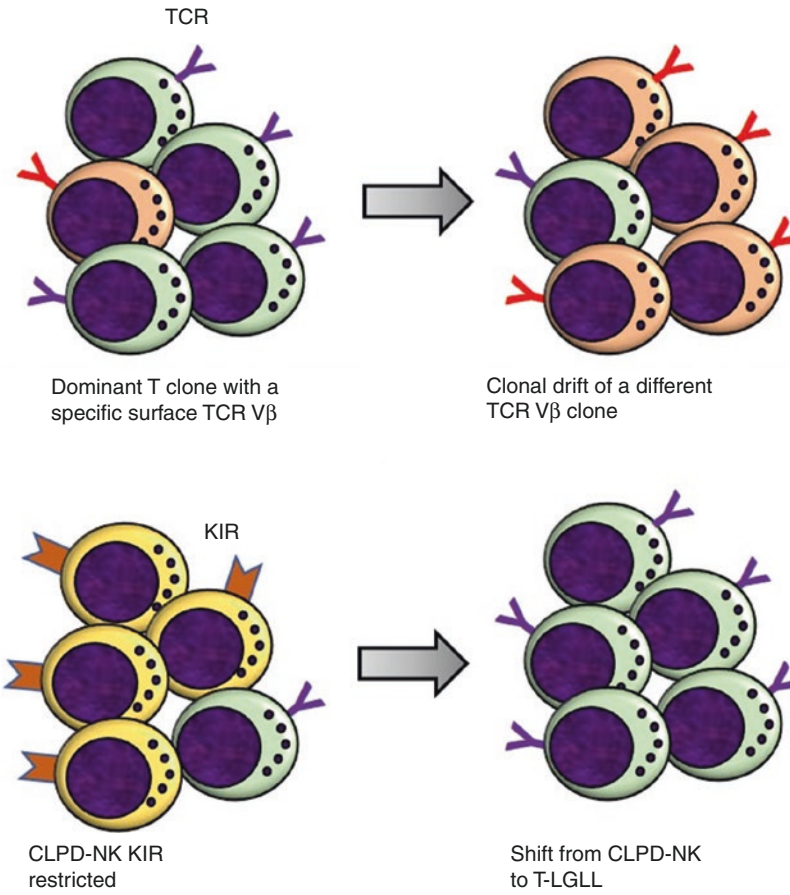

Fig. 15.5 Schematic representation of the main mechanisms leading to the phenomenon of clonal drift. *TCR* T cell receptor, *KIR* killer immunoglobulin-like receptor, *CLPD-NK* chronic lymphoproliferative disease of NK cells, *T-LGLL* T-large granular lymphocyte leukemia

selective tyrosine kinase inhibitor AG-490 or the STAT3-specific inhibitor, Stattic, *in vitro* induces LGL apoptosis highlights the role of STAT3 in LGL clonal expansion [66, 76]. In 2012, recurrent somatic mutations in the Src homology 2 (SH2) domain of the *STAT3* gene have been discovered. These gain-of-function mutations increase the stability of STAT3 dimers resulting in an enhanced transcriptional activity of the mutated proteins. Their frequency is 27–40% in T-LGLL patients [34, 77] and 9-30% in CLPD-NK [77, 90]. In the T-LGLL, *STAT3* mutations characterize the CD8+ T-LGLL and have never been observed in the CD4+ T-LGLL [35, 40, 89]. Patients with *STAT3* mutations present with neutropenia more frequently than patients without these mutations [35]. The association between PRCA and neutropenia with *STAT3* gene mutations was also reported in Asia [78, 79]. The most frequent *STAT3* mutations are Y640F and D661Y, representing 60% of the recognized mutations. Although almost all *STAT3* lesions are located in SH2 domain, very rare activating mutations were described also in DNA-binding and coiled-coil domains [80]. Another member of STAT protein family has been reported carrying activating mutations in the SH2 domain, *STAT5B*. *STAT5b* mutations were identified in 2% of the CD8+ T-LGLL, specifically affecting the rare aggressive form of LGL leukemia [39], and were also found in 15–55% of CD4+ T-LGLL, whereas indolent CD8+ T-LGLL and CLPD-NK seemed to be devoid of these genetic lesions [35, 40].

Since *in vitro* inhibition of STAT3 was observed to restore the apoptosis of LGL independently from *STAT3* mutational status and that STAT3 is activated also in *STAT3* wild-type LGLL patients, *STAT3* mutation cannot represent the only factor or be itself mandatory to

trigger LGL clonal expansion. In unmutated LGLL patients, a high amount of the proinflammatory cytokine IL-6 is able to activate the STAT3 axis [66], and the inhibition of this cytokine restores LGL apoptosis. Moreover, in LGLL the physiological negative feedback loop carried out by the suppressor of cytokine signaling (SOCS3) protein on the activated STAT3 is downregulated [66].

15.2.8 Other Cell Survival Dysregulated Pathways

Although STAT3 activation plays the most significant role in LGL disorders, other multiple cell survival pathways have been described. Increased activity of the PI3K-AKT signaling axis in T-LGLs appears to operate in conjunction with or parallel to increased STAT3 activation to inhibit the apoptotic program [81]. Sustaining this axis, RANTES, MIP-1beta, and IL-18 are high in serum LGLL patients [64]. Acting downstream of the PI3K-AKT pathway to prevent apoptosis through Mcl-1 independently of STAT3, a crucial role is played by NF-kB. Leukemic LGLs express high levels of c-Rel, a member of the NF-kB family, and exhibit higher NF-kB activity than normal PBMCs [70]. A pathogenetic role for NF-kB signaling is also proved by the finding of a recurrent (8%) mutation of TNFAIP3, a NFkB inhibitor [82]. In CLPD-NK, the activation of Ras/MEK/ERK pathway contributes to the accumulation of NK cells caused by a constitutive stimulation of both ERK and Ras. Consistently, Ras and ERK inhibition causes the reduction of the survival of patient NK cells [83]. In addition, ERK1/2 signaling can be activated also by a dysregulation of sphingolipid rheostat [84, 85].

15.2.9 Neutropenia Molecular Mechanism

The pathogenesis of neutropenia in these patients is probably multifactorial, including humoral and cytotoxic mechanisms. However, the most important mechanism accounting for neutropenia is myeloid progenitor and neutrophil destruction via Fas-mediated apoptosis [86, 87]. T-LGLs express Fas ligand (FasL), and mature neutrophils express Fas. There is high surface expression of Fas/FasL in LGL, and large amounts of soluble FasL have been detected in sera of neutropenic T-LGL patients [35, 86]. FasL and other inhibitory cytokines produced by LGL may lead to hematopoietic suppression through apoptosis of neutrophil precursors in the bone marrow or result in direct killing of neutrophils that express a high concentration of Fas on their surface. The correction of cytopenias in T-LGL leukemia has been associated with a disappearance or reduction of serum FasL levels [86]. Intriguingly, it has been reported that FasL expression can be driven by STAT3 activation (higher in *STAT3*-mutated patients) [35]. In addition, it was recently shown that a microRNA(miR)-146b is downregulated specifically in neutropenic patients so allowing the translation of human antigen R (HuR), an essential FasL mRNA stabilizer. HuR protein mediates FasL mRNA stabilization, leading to increased FasL production and, consequently, to neutropenia development [88].

15.3 Concluding Remarks

Further knowledge of developmental pathways of normal LGLs is crucial to get insights into the characteristics of LGL disorders. This would allow to face the urgent unmet need to develop better therapeutics for LGL leukemia, because this disease still remains incurable. The knowledge of the precise mechanism through which mutated STAT5b can mediate an aggressive disease in CD8+ T-LGLL whereas the same mutation when present in CD4+ T-LGLL does not induce detrimental effects is central to design targeted therapies for these patients. Due to the rarity of disease, well-designed prospective comparative clinical trials are up to now lacking. Only the results from a randomized clinical trial from the USA recruiting 55 patients are available showing a still unsatisfactory overall response rate [42]. In addition, the results of an important prospected randomized clinical trial from France

are coming (#NCT01976182). In the lack of this relevant information, the decision on which immunotherapy should be first started is still related to arbitrary medical decision. Finally, the possibility to know the biological mechanisms switching on and off in responding patients as compared to non-responding ones would certainly contribute to design more precisely targeted therapies.

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