

# Pathophysiologic Mechanisms And Management Of Large Granular Lymphocytic Leukemia Associated Pure Red Cell Aplasia

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**Abstract:** Large granular lymphocytic leukemia (LGLL) is a chronic clonal lymphoproliferative disease of mature T or NK cells, and produces a variety of hematological abnormalities. Pure red cell aplasia (PRCA) is a rare haematological disease and is one of the most common complications of LGLL. LGLL-associated PRCA may represent a relatively indolent type and may be more common than reported, but its natural history and clinical course have not been well described. The ethnic origin of the patients is an important consideration in determining the relationship between PRCA and LGLL. Guidelines and progresses for management of LGLL-associated PRCA rely on accumulation of empirical experiences, integrative analyses of several cases and clinical trials. The purpose of this review is to evaluate occurrence, possible mechanisms, diagnosis, clinical features, treatments and outcomes of LGLL-associated PRCA.

**Keywords:** large granular lymphocytic leukemia, lymphocytosis, PRCA

## Introduction

Large granular lymphocytic leukemia (LGLL) is a chronic clonal lymphoproliferative disease of mature T cells or NK cells, with most patients being asymptomatic and requiring no treatment during the course of the disease.<sup>1</sup> These conditions are thought to arise from chronic antigenic stimulation, while the long-term survival of the abnormal LGLs appears to be sustained by impaired survival signaling. LGLL produces a wide variety of hematological and immunological abnormalities<sup>1,2</sup> and pure red cell aplasia (PRCA) is one of the most common complications of LGLL in Asian patients.<sup>3,4</sup>

PRCA is a rare haematological disease, characterized by normocytic anemia, reticulocytopenia, and severe erythroid hypoplasia of bone marrow (BM).<sup>5</sup> PRCA includes an idiopathic form or a secondary form. Acquired secondary PRCA may be associated with thymoma, lymphoproliferative disorders, viral infections, solid tumors and a variety of other diseases.<sup>6,7</sup> There is a close association between LGLL and PRCA, and LGLL-associated PRCA accounts for a significant portion of secondary PRCA. Because LGLL is frequently underdiagnosed, it is likely that a significant proportion of idiopathic PRCA is secondary to LGLL and LGLL-associated PRCA may be more common than reported. T-LGLL is regarded to surpass all other pathologies as the most common cause of PRCA.<sup>8</sup> However, its natural history and clinical course have not been well described because of its rarity.

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### Occurrence

The incidence of LGLL-associated PRCA is 7.3–68.2%, as shown in Table 1. The age of the patients with LGLL-associated PRCA at diagnosis ranges from 18 to 89 years old and the LGL count ranges from 0.3 to 11.9×10<sup>9</sup>/L (Table 1). Oriental LGLL patients tend to present different features from occidental patients. In Asians, PRCA is the most common hematological complication of LGLL, while in western patients, rheumatoid arthritis (RA) and neutropenia are more frequent.<sup>6,9–13</sup> These differences might be partly due to ethnicity. So it is intriguing to explore the contributing factors, such as the genetic background.

The close association between T-LGLL and PRCA has been well described in both Japanese and Chinese patients. In 2 reports from Japan, 15 patients (42.9%) in one report met the criteria of PRCA among 35 T-LGLL patients and the incidence in the other was 11.0% (14/127).<sup>9,14</sup> In China, we reported PRCA was present in 18 (50%) of 36 T-LGLL patients and Zhao X et al reported 19 (67.9%) of 28 T-LGLL patients.<sup>10,15</sup> Kwong YL et al also reported PRCA was more frequent in Asians than western LGLL patients (52/110, 47% versus 6/143, 4%).<sup>16</sup>

In contrast, PRCA was less frequently observed in western LGLL patients. In the reports from western countries, LGLL-associated PRCA appeared in 15 (7%) of 203 patients and 6 patients (7.6%) were found to have PRCA in 201 T-LGLL patients.<sup>6,13</sup> In another report, Dhodapkar et al<sup>17</sup> identified 5 PRCA patients (7.4%) in 68 T-LGLL patients. In the latest study, Balasubramanian SK et al reported 14 PRCA patients (22%) in 62 LGLL patients.<sup>18</sup>

The LGLL seems to be triggered and sustained by antigenic drive, and hematopoietic progenitors may be major targets of this process.<sup>19</sup> Because antigen presentation depends not only on the peptide processing but also on HLA backgrounds, polarity of HLA distribution could affect the utilization of target molecules by LGLL. Therefore, the difference of HLA distribution between Asian and western populations may partially explain the distinction of the incidence and it would be important to know the ethnic origin of the patients. In oriental patients,

Table 1 Summary Of Articles About LGLL-Associated PRCA

No. Of Patients	Gender (M:F)	Median Age (years)	Rate Of LGLL (patients)	Rate Of PRCA (patients)	Median Number Of LGL (10 <sup>9</sup> /L)	STAT3 (+/Tested)	Reference
203 <sup>#</sup>	7:8	66 (28–88)	/	7.3% (15)	0.45 (range 0.3–7.21)	/	[6]
35 <sup>#</sup>	6:9	62 (34–81)	/	2.9% (15)	4.2 (range 0.7–11.9)	/	[9]
36 <sup>#</sup>	10:8	58.1 (38–89)	/	50.0% (18)	2.3 (range 0.9–6.7)	46.7%, 7/15	[10]
185 *	8:6	63 (44–85)	7.6% (14)	/	2.7 (range 0.96–4.8)	/	[12]
62*	9:5	68 (32–82)	22.6% (14)	/	2.7 (range 1.6–4.7)	45.5%, 5/11	[18]
42*	3:8	71 (20–78)	31.0% (13)	/	3.33 (0.87–5.51)	76.9%, 10/13	[59]
33 <sup>#</sup>	4:1	51 (47–73)	/	15.2% (5)	NR (range 0.68–11.6)	/	[64]
34 <sup>#</sup>	7:7	61 (25–78)	/	41.2% (14)	1.9 (range 0.4–5.3)	/	[71]
22 <sup>#</sup>	12:3	50 (40–84)	/	68.2% (15)	2.1 (range 0.8–11.6)	/	[16]
42 <sup>#</sup>	NR	NR	/	45.2% (19)	NR	30.9%, 13/42	[31]

Notes: \*These are PRCA patients. #these are LGLL patients. Abbreviation: NR, not reported.

an underlying LGLL should be excluded in the diagnostic evaluation of PRCA and different clinicopathologic and epidemiologic factors may be involved.

## Possible Mechanisms

Are LGLs causing PRCA, or is their presence more likely to be an epiphenomenon as a consequence of chronic antigen stimulation? The pathophysiological mechanism of LGLL-associated PRCA has not been evaluated and proper distinction of pathogenesis is essential for selecting effective therapeutic modality. Multiple mechanisms have been postulated, including inhibition of erythropoiesis by LGLs, chronic antigenic stimulation due to viral infections, autoimmune diseases, humoral and cytotoxic mechanisms.<sup>20,21</sup>

## Viral Infections

LGLL-associated PRCA may be associated with long-term antigenic stimulation due to viral infections, especially parvovirus B19, which may result in LGL lymphocytosis and may play an etiologic role in some PRCA patients. Parvovirus B19 infection has been reported to be associated with PRCA and there are several reports of T-LGLL in response to parvovirus B19 infection.<sup>21–24</sup>

Ergas D et al reported parvovirus B19 infection in 3 LGLL-associated PRCA patients, which showed a cross-link between parvovirus B19 infection and the appearance of PRCA.<sup>21</sup> These patients had had years of LGLL with a stable hemoglobin level, but PRCA developed after the occurrence of parvovirus B19 infection. Some patients with parvovirus B19-induced PRCA have been reported to have had LGLL.<sup>24</sup> LGL expansion could have been initiated after parvovirus B19 infection. There have been other reports of LGLL-associated PRCA that developed after parvovirus B19 infection. In some LGLL-associated PRCA patients, parvovirus B19 was found to be positive after diagnosis.<sup>18</sup> Kondo H et al reported a T-LGLL patient developed parvovirus B19-associated PRCA.<sup>25</sup> These observations suggest that LGLL patients are at an increasing risk to develop parvovirus B19 infection and underscore the need to screen routinely for this infection. LGLs in the peripheral blood should be carefully examined in patients with parvovirus B19-induced PRCA, and LGLL patients who exhibit anemia should be evaluated for parvovirus B19 infection.

A direct toxic effect of parvovirus B19 on pronormoblasts may result in PRCA. Parvovirus B19 directly infects erythroid progenitors through the red cell surface P antigen. Individuals whose erythroid progenitors do not express P antigen are

resistant to parvovirus B19 infection.<sup>26–28</sup> Parvovirus B19 has been identified in proliferating CFU-E and serum that contains parvovirus B19 inhibits CFU-E growth in vitro, and this effect can be inhibited by antibody to this virus.<sup>29,30</sup> To find out whether parvovirus B19 is related with LGLL, additional studies are warranted to address the role of parvovirus B19 in the pathogenesis of LGLL.

## LGLs-Mediated Inhibition Of Erythropoiesis

PRCA may be mediated by LGLs against erythroid progenitors.<sup>20,23,31–39</sup> The mechanism of inhibition may operate at different levels of erythroid differentiation and even be genetically restricted.<sup>34,40</sup> This is likely via LGL-mediated killing of erythroid colony units (CFU-E) and burst forming units (BFU-E), thereby inhibiting erythroid precursor progression to mature erythrocytes.<sup>20,31,34,35</sup>

The T-LGLs can mediate direct cellular cytotoxicity and inhibit erythroid cell differentiation. It has been demonstrated that LGLs inhibited erythroid hematopoiesis with co-culture techniques in vitro. Hara T et al demonstrated that the LGLs from a LGLL-associated PRCA patient inhibited BFU-E in culture.<sup>33</sup> Abkowitz et al demonstrated T-LGLs from PRCA patients mediated inhibition of erythropoiesis in marrow culture systems.<sup>34</sup> In another report, T-LGLs inhibited the growth of erythroid colonies in vitro and CFU-E growth increased markedly after removal of the T-LGLs.<sup>37</sup> Nagasawa T et al investigated the interaction between CFU-E and the LGLs in vitro.<sup>36</sup> The CFU-E was strongly suppressed when the LGLs were cocultured with normal allogeneic BM cells and the LGLs frozen before treatment also suppressed CFU-E of autologous BM cells after the recovery from PRCA. These demonstrated directly that the LGLs were capable of suppressing erythropoiesis. In contrast, erythroid progenitors were significantly increased when LGLs were removed. In vitro, growth of the patient's erythroid progenitors has been accomplished either after removal of LGLs or after induction of remission.<sup>41</sup> These results indicated that the interaction between the T-LGLs and hematopoietic stem cell may be important for the mechanism of erythroid suppression. That T-LGLs are able to suppress erythroid hematopoiesis is consistent with the observations that T-LGLs may be causative in aplastic anemia.<sup>42,43</sup> Furthermore, these results obtained from in vitro coculture studies were consistent with the clinical findings.

NK cells might be involved in the physiological regulation of haematopoiesis. A possible pathogenetic impact

of hyperactive NK cells should be considered in acquired PRCA. As with T cells, NK cells may have a negative regulation on erythropoiesis and can suppress CFU-E in vitro.<sup>38,44</sup> Normal human thymus and BM contain immature hematopoietic cells which are sensitive to NK-cell-mediated cytotoxicity in vitro.<sup>45-47</sup> Partanen S et al reported NK cells from PRCA patients inhibited BFU-E and CFU-E in vitro and showed a very high NK activity against the human erythroblastic leukemia cell line K562.<sup>38</sup>

## Human Leukocyte Antigen (HLA) Class I Expression

The reduced expression of HLA class I genes in the erythropoietic lineage accounts for the selective cytotoxicity of LGLs against erythroid progenitors. KIRs (Killer-cell immunoglobulin-like receptors) on the LGLs bind to HLA class I molecules and then prevent LGLs to cause cytolysis.<sup>47</sup> HLA class I molecules are expressed by erythroid precursors, whereas mature erythroid precursors have reduced the expression of HLA class I molecules.<sup>48,49</sup> LGLs expressing KIRs can destroy erythroid precursors because of the physiologic down-regulation of HLA class I molecules. The reduced expression of HLA class I molecules prevents the transmission of adequate inhibitory signals, which enhances major histocompatibility (MHC)-unrestricted cytotoxicity of LGLs and allows LGLs to cause cytolysis.<sup>47</sup> Erythroid precursors become sensitive to MHC-unrestricted cytolysis as the expression of HLA class I molecules is reduced.

NK cells express KIRs and the repertoire of KIRs differs from person to person. Like NK cells, cytotoxic  $\gamma\delta$  T cells also express KIRs, suggesting that both NK cells and  $\gamma\delta$  T cells survey the body for missing self HLA class I molecules. Handgretinger R et al reported the  $\gamma\delta$ T-LGLs inhibited erythroid precursors by KIRs in a patient with  $\gamma\delta$ T-LGLL-associated PRCA.<sup>50</sup> These  $\gamma\delta$ T-LGLs expressed KIRs and killed erythroid progenitors in an MHC-unrestricted manner. Although most LGLs were of the T cell receptor (TCR)  $\alpha\beta$ -type, some  $\alpha\beta$ T-LGLs express the KIRs.

## Immune Mechanisms

Immune mechanisms contribute to the pathogenesis of LGLL-associated PRCA as indicated in favourable response to immunosuppressive treatment.<sup>5,36</sup> LGLL-associated PRCA develops due to autoantibody-dependent immune mechanisms and has been shown to be associated with immunoglobulins.<sup>50</sup> These LGLs have exhibited surface Fc

receptors for IgG and suppressed immunoglobulin biosynthesis by B cells.<sup>36</sup> IgG antibodies directed against erythroid precursors have been demonstrated in some cases.<sup>51,52</sup> Antibodies against erythroid cells or erythropoietin can cause PRCA.<sup>53</sup> LGLs activation is dependent on the antibody against erythroid precursors.<sup>47</sup>

## Molecular Mechanism

Genetic changes might lead to the formation of LGLL. STAT3 mutations resulting in persistent proliferation of LGLs are a frequent event in LGLL and aberrant STAT3 signaling underlies the pathogenesis.<sup>54-56</sup> LGLL-associated PRCA may also involve activation of the STAT3 pathway. However, studies evaluating this pathway in LGLL-associated PRCA are lacking.

STAT3 mutations were frequently present in PRCA patients, irrespective of the etiology. STAT3-mutated T-cell subclones were found in a subset of PRCA, suggesting contributions of STAT3-mutated T cells in this disorder.<sup>57</sup> STAT3 mutations were also frequently present in LGLL-associated PRCA patients.<sup>31,58</sup> Significant associations between STAT3 mutations and PRCA further imply their roles in PRCA. STAT3 mutations were found in 5 of 15 patients with idiopathic PRCA (33%), and 10 of 13 patients with T-LGLL-associated PRCA (77%).<sup>18</sup> Among the PRCA patients, 3 of the 29 PRCA patients without T-LGLL and 6 of the 13 PRCA patients with T-LGLL were positive for STAT3 mutations.<sup>59</sup> These results suggested that the STAT3 mutations were limited to PRCA patients with or without T-LGLL. LGLL-associated PRCA displayed a significant association with STAT3 mutation. PRCA with STAT3 mutation was recognized in 13 T-LGLL patients.<sup>31</sup> In our research, 7 patients with LGLL-associated PRCA (25%) were found to have STAT3 mutations.<sup>10</sup> Balasubramanian et al recently reported the results of targeted sequencing for PRCA patients with and without T-LGLL.<sup>18</sup> They detected STAT3 mutations in 5 of 11 T-LGLL-associated PRCA patients but not in any of 17 patients with idiopathic PRCA. But Jerez A et al could not detect STAT3 mutations in patients with LGLL-associated PRCA (0/48) and PRCA were invariably related to STAT3 wild-type patients (6%, 8/122).<sup>55</sup> Shi et al also reported that PRCA was seen exclusively in the STAT3 wild-type T-LGLL patients, and patients with STAT3 mutations were more prone to develop RA.<sup>60</sup> The absence of STAT3 mutations may be due to the ethnic background of the patients. T-LGLL is known to be frequently accompanied by RA or neutropenia in western

countries, whereas PRCA is frequently associated with T-LGLL in East Asian populations.<sup>10,12,16</sup>

However, it is still unclear whether STAT3-mutated T cells have an inhibitory effect on erythroid cell production or not. Because STAT3 mutations could develop in T cells that were chronically stimulated by inflammatory cytokines or various antigens, the presence of STAT3-mutated T cells may be an epiphenomenon rather than pathogenic.<sup>61,62</sup> The mutational process in the STAT3 gene and the functional aspects of STAT3-mutated T cells thus require further investigation.

## Cytokines

Cytokines released by LGLs, such as tumor necrosis factor (TNF) which is known as inhibitors of hematopoiesis, may be responsible for the PRCA in LGLL. The patients with untreated PRCA showed high levels of IL-6, IL-8, IL-10, TNF $\alpha$  and sIL-2R, while patients with PRCA treated with immunosuppressive agents showed low levels of TNF $\alpha$  and sIL-2R.<sup>63</sup> Interferon- $\gamma$  production by T-LGLs may have mediated the suppressive effects of erythropoiesis.<sup>7</sup>

## Diagnosis

PRCA was recognized in conjunction with or shortly after LGLL diagnosis. But the diagnosis of LGLL-associated PRCA is still challenging. The currently diagnostic criteria do not distinguish a idiopathic PRCA or a secondary PRCA. It is important to separate the categories of PRCA, because immunosuppression will not only be ineffective in clonal myelopoietic disorders but also may even accelerate transformation.

Based on the published literature, there are several important considerations in making a diagnosis of LGLL-associated PRCA: (1) the lymphocyte count is usually  $2\text{-}20\times 10^9/\text{L}$  and LGL counts  $> 2\times 10^9/\text{L}$  (when LGL counts  $< 2\times 10^9/\text{L}$  diagnosis is confirmed by the detection of clonality); (2) the predominance of LGLs in the peripheral blood smear samples; (3) the clonality of LGL lymphocytosis; (4) the phenotypes of LGLs are usually CD2+CD3+CD8+CD57+ T-cell or surfaceCD3-cCD3epsilon+CD16+ NK-cell; (5) PRCA was defined as isolated anemia (untransfused Hb  $< 8\text{g/dL}$ ) with an absolute number of reticulocyte  $< 2.0\times 10^4/\text{L}$  ( $< 1\%$ ) or BM erythroblasts  $< 10\%$  of all nucleated cells;<sup>64</sup> (6) Other known causes of PRCA were excluded.

The BFU-E assay can distinguish among MDS, systemic autoimmunity, LGLL and PRCA, which may be an important adjunct to the work-up of the undiagnosed

cytopenias.<sup>65</sup> Low BFU-E growth ( $< 10\text{ BFU-E}/10^5$  marrow mononuclear cells) is more consistent with a diagnosis of MDS and helps to exclude the diagnosis of PRCA, LGLL, or cytopenias from autoimmune diseases. More robust BFU-E growth ( $\geq 20\text{ BFU-E}/10^5$  marrow mononuclear cells) is usually associated with external suppression hematopoiesis such as occurs in LGLL, PRCA, or autoimmunity. BFU-E growth for patients with LGLL-associated PRCA was higher than that of patients with non-LGLL-associated PRCA. Growth above the normal median ( $40\text{ BFU-E}/10^5$  marrow mononuclear cells) always excluded MDS and was also associated with response to IST.

It is important for hematopathologists and hematologists to be aware of this entity in order to guide treatment strategies. We propose a diagnostic algorithm for the diagnosis of LGLL-associated PRCA (Figure 1).

## Clinical Presentation

There is no specific clinical presentation of LGLL-associated PRCA and the signs and symptoms are those associated with anemia, such as fatigue, pallor, palpitations, peripheral neuropathy and shortness of breath on exertion.<sup>64,66</sup> Because PRCA is a pure underproduction anemia, the gradual decline in hemoglobin concentration allows some degree of adaptation, and symptoms may be fewer than would be expected for the degree of anemia.

Comparing STAT3 mutation(+) LGLL-associated PRCA patients with STAT3 mutation(-) patients, the STAT3 mutation(+) patients had more lymphocytes, more CD8+ T cells<sup>59</sup> and showed a trend towards a younger age (median age, 52.5 years old and 77.5 years old) than the STAT3 mutation(-) patients.<sup>31</sup> LGLL-associated PRCA patients showed a higher frequency of T-cell clonality and STAT3 mutations than PRCA patients without T-LGLL.<sup>59</sup>

Some other diseases have been associated with LGLL-associated PRCA: thymoma,<sup>6,67</sup> polyglandular autoimmune syndrome,<sup>36,68</sup> chronic lymphocytic leukemia, non-Hodgkin lymphoma and amegakaryocytic thrombocytopenic purpura,<sup>68,69</sup> but it is notable that RA and neutropenia were infrequently associated.

## Types Of LGL Lymphocytosis

PRCA-associated LGL lymphocytosis typically comprises monoclonal or oligoclonal expansion of cytotoxic T- or NK-cells. Reactive LGL lymphocytosis is usually polyclonal, transient, and asymptomatic. Neoplastic clonal LGL expansions are classified as T-LGLL and CLPD-NK.

## Reactive LGL Lymphocytosis

Some PRCA patients have elevated levels of LGLs but do not fulfill the diagnostic criteria for LGLL.<sup>23</sup> In these patients, the LGL lymphocytosis may be polyclonal and may rarely evolve into LGLL.<sup>22,23,70</sup>

## T-LGLL

T-LGLL is most frequent in LGLL-associated PRCA, and has been reported in case report or case series.<sup>6,9,10,12,16,18,59,64,71</sup> These LGL expansions have been defined as neoplastic processes because of their clonal nature. Most T-LGLL is of the  $\alpha\beta$  type, but some cases of T-LGLL were of the  $\gamma\delta$  type.<sup>72,73</sup>  $\gamma\delta$ T-LGLL is rare, and its association with PRCA is also rare.  $\gamma\delta$ T-LGLL-associated PRCA was first described by Oshimi K et al.<sup>74</sup> Tanaka et al reported a case of  $\gamma\delta$ T-LGLL complicated with PRCA at presentation, and later with Epstein-Barr virus (EBV) infection.<sup>75</sup> The disease was first diagnosed as PRCA, but  $\gamma\delta$ T-LGLL developed later, and PRCA was retrospectively considered to be caused by  $\gamma\delta$ T-LGLL. Handgretinger R et al also described a patient with  $\gamma\delta$ T-LGLL-associated PRCA.<sup>50</sup> In Japan,  $\alpha\beta$ T-LGLL and  $\gamma\delta$ T-LGLL were frequently associated with PRCA.<sup>3,76</sup>

Although several theories have been proposed, the mechanism by which PRCA develops in  $\alpha\beta$ T-LGLL and  $\gamma\delta$ T-LGLL is not well understood.  $\gamma\delta$ T-LGLL is strongly suspected when LGLs show CD3+CD4-CD8- phenotype.

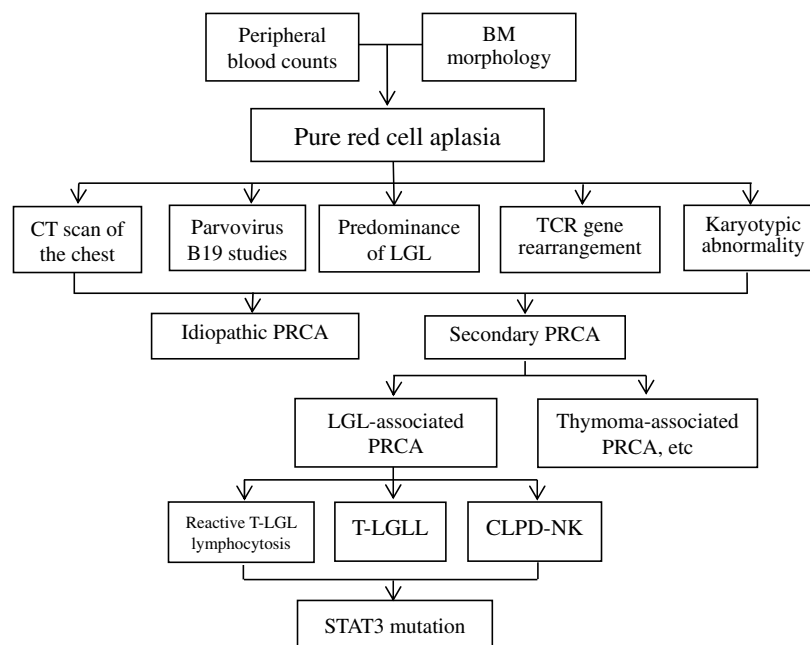
However, the incidence of PRCA (29%) in CD4-/CD8-  $\gamma\delta$ T-LGLL was much higher than that reported in CD8+  $\gamma\delta$ T-LGLL (6%),<sup>77,78</sup> and the incidence of PRCA in  $\gamma\delta$ T-LGLL (29%) was also higher than that reported in  $\alpha\beta$ T-LGLL (7%).<sup>17</sup> However, the higher incidences of PRCA need to be confirmed by accumulation of data from a larger number of cases of CD4-/CD8-  $\gamma\delta$ T-LGLL. The higher incidences of PRCA observed in CD4-/CD8-  $\gamma\delta$ T-LGLL may be related to the higher cytotoxic activity observed in the CD5- subset of  $\gamma\delta$ T cells than that in the CD5+ subset.<sup>79</sup>

## CLPD-NK

CLPD-NK is a rare type in secondary PRCA and has been reported in case report.<sup>3,38,39,50</sup> Tefferi A et al reported a case of CLPD-NK-associated PRCA and the tumour cells expressed features consistent with NK cells, but EBV was absent from the tumour cells.<sup>39</sup> In 1994, Tefferi A et al reported another case of CLPD-NK-associated PRCA.<sup>80</sup> Kurt H et al also reported a patient with PRCA in 11 CLPD-NK patients.<sup>81</sup>

## Clonality And PRCA

PRCA is mediated by a spectrum of T cell responses ranging from polyclonal to oligo and monoclonal. The presence of STAT3 mutations may support this notion,<sup>23</sup> as STAT3 mutation(+) LGLs are usually a sub fraction of



**Figure 1** Diagnostic algorithm for the differential diagnosis of LGLL-associated PRCA.

**Abbreviations:** BM, Bone marrow; PRCA, pure red cell aplasia; LGL, Large granular lymphocyte; TCR, T-cell receptor.

clonal cytotoxic T lymphocytes (CTL). Reactive T lymphocytosis can evolve from polyclonal to clonal and be further fixed by STAT3 mutations.<sup>13</sup>

High frequencies of clonal TCR gene rearrangements were observed in PRCA patients. Balasubramanian SK et al reported 8 (25%) of the 32 patients were positive for clonal TCR gene rearrangement but did not fulfill the criteria for LGLL.<sup>18</sup> Sivakumaran et al reported two PRCA patients had clonal TCR gene rearrangements.<sup>82</sup> LGLL-associated PRCA patients had clonal TCR gene rearrangements more often than the PRCA patients (65% vs. 33%, respectively).<sup>15</sup> In a report from oriental patients, 19% PRCA patients with TCR gene rearrangement were diagnosed as T-LGLL and the actual frequency might be higher as only some of the patients had been investigated.<sup>20</sup> The significance of this observation is that in oriental patients, and perhaps also in occidental patients, an underlying T-LGLL should be excluded in the diagnostic evaluation of PRCA. Motoji et al reported the TCR gene rearrangement could not be detected after a LGLL-associated PRCA patient was treated successfully.<sup>83</sup> The significance of this observation is that disappearance of TCR gene rearrangement is an effective indicator of treatment.

## Treatments

LGLL-associated PRCA is an orphan disease without rationally established standard treatments and optimal treatment is controversial. Treatment is mainly based on retrospective case series, as well as on case reports, however, responses are unpredictable, and a sizable proportion of patients may be refractory and transfusion dependent. Several agents, such as methotrexate (MTX), cyclosporine (CsA), cyclophosphamide (CTX), prednisone, hematopoietic growth factors and nucleoside analogs have been used with variable success,<sup>60,61</sup> and alemtuzumab and antithymocyte globulin have been proposed for refractory disease.<sup>62,63</sup> The goal of treatment is to attain a normal hemoglobin concentration or transfusion independence with clinically acceptable hemoglobin concentration.

### Methotrexate (MTX)

Based on the data previously discussed, first-line treatment includes low-dose MTX with or without steroids. MTX is usually administered orally at a weekly dose of 5–13.5 mg/m<sup>2</sup>. A trial of at least 3 months is necessary before MTX therapy is characterized as ineffective. The efficacy of MTX as first-line treatment was evaluated on 10 LGLL-associated PRCA patients.<sup>18</sup> The overall CRR

(complete response rate) and ORR (overall response rate) was 20% (2/10) and 30% (3/10), respectively. Our series of Chinese T-LGLL-associated PRCA patients responded well to MTX.<sup>10</sup> In the 18 patients with T-LGLL-associated PRCA there were 6 complete and 11 partial responses with an ORR of 94%. Median time-to-response was 3 months (range, 1–5 months). Response rate in PRCA patients was not significantly different from that in all other patients.

Treatment should be continued indefinitely in responders. Given the potential marrow-suppressive effect of MTX, its use should be monitored in patients with hepatic and renal function and avoided in patients with impaired hepatic or renal function. MTX interferes with the cellular utilization of folate, and folate depletion is the cause of most of the side effects. So low-dose oral folate (1–5 mg daily) should be given to prevent side effects. But folate may reduce the efficacy of MTX.

### Cyclophosphamide (CTX)

CTX at doses of 50–100 mg orally daily, is effective for inducing remission and preventing relapse in LGLL-associated PRCA.<sup>6,12,14,84</sup> There were 4 case series that reported LGLL-associated PRCA showed better outcomes with CTX with response rates of 60–100% when compared with CsA or steroids.<sup>6,12,84,85</sup> As an initial therapy, CTX produced a better ORR than prednisone alone (80% versus 43%) and was associated with a longer duration of response, 60 months (range, 22–117 months) versus 7.5 months (range, 5–43 months).<sup>6</sup> Five patients received CTX (4 CR, 1 NR), the remission duration ranged from 22 to 120 months.<sup>6</sup> Fujishima et al have demonstrated that the ORR to CTX is 75% (2 CR and 4 PR) and the median relapse-free survival was 53 months in LGLL-associated PRCA.<sup>12</sup> CTX seemed to have a better activity in LGLL-associated PRCA than CsA, but this was not statistically significant.<sup>12</sup> The median duration of CTX therapy was 24 months with a range of 10–124 months.<sup>12</sup> The ORR to CTX was 47% (14/30) and there was no difference in ORR between the LGLL and LGLL-associated PRCA group.<sup>18</sup> The ORR to the initial CTX therapy was 66% and the median duration of response was 32 months.<sup>85</sup> In 7 patients with T-LGLL-associated PRCA, all of them were successfully treated with CTX monotherapy.<sup>84</sup> Therapeutic responses began after 8 weeks, and clinical CRs were obtained after 6 months. Clinical remission was associated with the disappearance of TCR gene rearrangement, which suggested that the disappearance of TCR gene rearrangement might be

an indicator for the discontinuation of CTX. Hansen RM et al reported a LGLL-associated PRCA patient who experienced a partial and then a complete clinical response to splenectomy and CTX.<sup>41</sup> CTX was cytotoxic not only for T-LGLL, but also the polyclonal proliferation of T-LGLs. Rie Tabata et al showed that CTX was effective when PRCA was caused by increased T-LGLs.<sup>86</sup> The treatment had to be maintained to achieve continued remission, but CTX was of limited value as a maintenance agent due to its late toxicity.

The precise underlying mechanism is still unknown, and one possible explanation is that CTX is effective to reduce the CTLs, which damaged antibody-bound erythroblasts directly. On the other hand, CTL-mediated damage to hematopoietic progenitors could cause MDS, which lead to anemia, and reduction of CTLs by CTX could avoid this phenomenon.

## Cyclosporine (CsA)

LGLL-associated PRCA also showed a good response to CsA, which may be proposed as first line therapy.<sup>14,15,87,88</sup> In cases not responding to MTX, CsA may be administered as a second-line therapy. A reasonable starting dose is 5–10 mg/kg per day orally in two divided doses for at least 3 months. Fujishima N et al reported CsA produced remissions in 1/4 patients. 5 patients were maintained on CsA and 2 patients relapsed during maintenance therapy.<sup>12</sup> In a report, response rate for T-LGLL with or without PRCA was analyzed.<sup>15</sup> The 19 patients with T-LGLL-associated PRCA achieved an ORR of 78.9% (11 CR, 4 PR), while 9 patients without PRCA achieved an ORR of 88.9% (5CR, 3PR). In a study on 25 LGLL-associated PRCA patients, the ORR was 56% (28% achieved CR).<sup>89</sup> ORR was better in non-LGLL vs LGLL-associated PRCA (84% vs. 55%,  $P=0.01$ ).<sup>18</sup> Kawakami T et al reported that the response rate to CsA in the STAT3 mutation(+) PRCA patients was significantly lower than that in the STAT3 mutation(-) patients (46% [6 of 13] vs 100% [8 of 8]).<sup>59</sup> Two STAT3 mutation(+) patients acquired resistance to CsA, whereas 8 STAT3 mutation(-) patients remained responsive to CsA.

CsA is an immunosuppressive agent which seems to alter T-cell function by inhibiting the production of IL-2 from the helper T cells. One of the advantages of CsA is its rapid effect; the disadvantages of CsA are renal toxicity, hepatotoxicity, gastrointestinal complication, hirsutism, hypertension and so on, but these effects are dose-dependent and reversible. CsA levels should be monitored with

target levels of 150 to 250 ng/mL.<sup>87</sup> After achieving remission, CsA can be tapered slowly to the lowest effective maintenance dose.

## Steroids

Prednisone is usually administered at a dose of 40–60 mg/day and appears to be useful when used in combination with immunosuppressive agents. Single-agent steroid therapy was of limited value and may be associated with unwanted toxicity. Some patients may have a response, but most of them were not durable responses. Go RS et al reported 7 patients were treated with prednisone (3 CR, 4 NR), but all prednisone responders relapsed within 5 to 10 months of treatment.<sup>6</sup>

## Alemtuzumab

The humanized anti-CD52 antibody alemtuzumab (Campath-1H) is capable of selectively killing CD52-expressing cells and might be suitable for LGLL-associated PRCA because it is effective in not only T-cell lymphoproliferative diseases,<sup>90,91</sup> but also PRCA.<sup>87</sup> Alemtuzumab should be used with caution in selected cases that are refractory to other treatments. Alemtuzumab is given at a dose of 10 mg/week for 4–6 weeks, and response is assessed with careful monitoring of the blood counts. Successful outcomes with alemtuzumab in the salvage setting have been described.<sup>89,92,93</sup> Alemtuzumab showed promising results in LGLL-associated PRCA with an ORR of 75% and overall CRR of 63%.<sup>18</sup> It was highly efficacious for 2 patients with LGLL-associated PRCA.<sup>94</sup> The mean response time was 17 days compared with a response time of at least 61 days on standard first-line therapy. Wing-Yan Au et al reported treatment with alemtuzumab led to a complete remission of the PRCA in a T-LGLL patient.<sup>93</sup> A patient with thymoma, PRCA and T-LGLL was treated with alemtuzumab and there was an increase in haemoglobin but leukaemic remission did not occur.<sup>95</sup> Therefore, this observation suggested that alemtuzumab might not be curative for T-LGLL. This response of PRCA to alemtuzumab independent of the leukaemia was reminiscent of that observed when CsA was used to treat T-LGLL,<sup>96</sup> where improvement of cytopenias occurred despite persistence of the leukaemia. Schützinger C et al reported successful treatment with alemtuzumab in a patient with  $\gamma\delta$ T-LGLL-associated PRCA refractory to CsA and MTX.<sup>92</sup> This case showed that alemtuzumab was highly effective for treatment-resistant  $\gamma\delta$ T-LGLL and led to a rapid and sustained elimination of PRCA.

Infection is an important complication of alemtuzumab. Virustesting should be carried out before treatment.



In a report, 56% patients developed opportunistic infections, and herpesvirus infections were the most common.<sup>97</sup> Other infections included upper respiratory tract infections, tuberculosis, sepsis and/or bacteraemia.<sup>97</sup>

## Antithymocyte Globulin

Antithymocyte globulin has a 50% response rate in primary autoimmune PRCA, but has relatively low response rate in refractory PRCA. LGLs inhibited normal BM CFU-E growth, and this effect could be abolished by treatment with antithymocyte globulin.<sup>42</sup> A T-LGLL-associated PRCA patient was refractory to treatment, but responded to antithymocyte globulin and became transfusion independent.<sup>64</sup> In two patients with T-LGLL-associated PRCA, administration with antithymocyte globulin resulted in CR in one patient, whereas the other patient was refractory to all types of treatment and remained transfusion-dependent.<sup>21</sup>

Notably, there should be a therapeutic trial for at least 2 to 3 months before a specific agent is deemed ineffective. Although CTX, MTX and CsA are effective treatment, they have also been shown to increase the incidence of secondary malignancies. This is thought to be related to the immunosuppressive effect. In some cases, molecular remission was achieved, although this was not a prerequisite for long-term clinical remission. Remissions can be achieved in most cases with sequential immunosuppressive treatment. In about 5% cases, there was no response to several lines of treatment and chronic red cell transfusion was necessary. More intensive cytotoxic treatment such as combination chemotherapy is usually unnecessary. The cumulative dose of the medication and the duration of the maintenance treatment is one of the major concerns considering the late toxicity. Therefore, strategies that reduce the duration of exposure can minimize the long-term risks.

In refractory cases, study of newer immunosuppressive agents may provide new treatment options. Gene signature and mutational profiling may be an effective tool in determining what an appropriate treatment is. Patients with STAT3 mutations also tended to need more treatments.<sup>59</sup> In LGLL-associated PRCA patients with STAT3 mutation, STAT3 activation led to multiple gene deregulations and treatment with STAT3 inhibitors induced the apoptosis of leukemic cells.<sup>56</sup> As a result, specific STAT3 inhibitors have been evaluated as a novel therapeutic regimen for LGLL-associated PRCA. These observations were not limited to the STAT3 mutation(+) patients with LGLL-associated PRCA; similar findings were also recognized in STAT3 mutation(-) patients with LGLL-associated

PRCA, but not in normal controls.<sup>98,99</sup> The mechanisms of proliferative and/or anti-apoptotic characteristics of LGLs in STAT3 mutation(+) patients might be similar to those in STAT3 mutation(-) patients through alternative changes other than STAT3 SH2 domain mutations.

## Prognosis

LGLL is the disease commonly associated with PRCA. This association predicts superior response to immunosuppressive treatment, but is not correlated with improved survival. The patients with LGLL-associated PRCA had a better response than those with idiopathic PRCA (88% v 56%).<sup>20</sup> However, this result did not reach statistical significance, and the survival time was not significantly different between the two groups.<sup>20</sup> The median overall survival of idiopathic PRCA was 212.6 months, and the median overall survival of LGLL-associated PRCA was 147.8 months.<sup>14</sup> Survival time was not significantly different between the idiopathic PRCA and LGLL-associated PRCA. Response of patients with relapsing PRCA to immunosuppressive treatment was inferior to those of untreated PRCA patients (65.8% vs 80%).<sup>14</sup> We sought to identify potential prognostic factors and to review response rates to various treatment regimens. Patients with STAT3 mutation had briefer treatment-free survival than patients with wild-type STAT3 (5 months [range, 0.5–13 months] vs 19 months [range, 3–97 months]). Time-to-response and response-duration were also briefer in STAT3 mutation patients but these differences were not significant.<sup>58</sup> Infection and organ failure, but not progression of LGLL, were the principal causes of death. Refractoriness to induction immunosuppressive treatment and relapse of anaemia may be risk factors for death in LGLL-associated PRCA. Effective maintenance treatment, treatment of post-transfusion iron overload and the management of infectious complications are crucial for improving the prognosis of LGLL-associated PRCA.

## Conclusion

LGLL remains an underdiagnosed disorder. A significant proportion of idiopathic PRCA may be secondary to LGLL. T-LGLL is the disease most commonly associated with PRCA in Asian patients. Immunosuppressive treatments have efficacy in LGLL-associated PRCA and distinction of pathogenetic mechanisms is essential for selecting the most effective therapeutic modality. As the pathogenetic mechanisms of LGLL-associated PRCA become elucidated, more targeted and less toxic treatments

are expected to be developed. Nevertheless, comparison of these studies is limited by unavoidable heterogeneity in terminology, diagnostic criteria and patient populations.

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## Disclosure

The authors report no conflicts of interest in this work.

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