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Large granular lymphocyte leukemia: from dysregulated pathways to therapeutic targets

Francis LeBlanc¹, Dan Zhang¹, Xin Liu¹, and Thomas P Loughran Jr.^{*1}

¹Penn State Hershey Cancer Institute, Experimental Therapeutics, Room 4427, 500 University Drive, PO Box 850, Hershey, PA 17033-0850, USA

Abstract

Large granular lymphocyte (LGL) leukemia is a clonal lymphoproliferative disorder of cytotoxic lymphocytes characterized by an expansion of CD3⁺ cytotoxic T lymphocytes or CD3⁺ natural killer cells. Patients present with various cytopenias including neutropenia, anemia and thrombocytopenia. In addition, there is an association of T-cell large granular lymphocytic leukemia with rheumatoid arthritis. It is believed that LGL leukemia begins as an antigen-driven immune response with subsequent constitutive activation of cytotoxic T lymphocytes or natural killer cells through PDGF and IL-15 contributing to their survival. Consequently, this leads to a dysregulation of apoptosis and dysfunction of the activation-induced cell death pathway. Treatment of LGL leukemia is based on a low-dose immunosuppressive regimen using methotrexate or cyclophosphamide. However, no standard of therapy has been established, as large prospective trials have not been conducted. In addition, some patients are refractory to treatment. The lack of a curative therapy for LGL leukemia means that new treatment options are needed. Insight into the various dysregulated signaling pathways in LGL leukemia may provide novel therapeutic treatment modalities.

Keywords

large granular lymphocyte; leukemia; lymphoid leukemia; therapeutic intervention

Large granular lymphocyte (LGL) leukemia is a rare lymphoproliferative disorder of cytotoxic lymphocytes. In normal adults, LGLs comprise 10–15% of peripheral blood mononuclear cells (PBMCs). The LGLs consist of two distinct subpopulations: CD3⁺ cytotoxic T lymphocytes (CTLs) and CD3⁺ natural killer (NK) cells. These two lineages play major roles in the immune system. After activation through antigen recognition, LGLs undergo substantial expansion and normally die by apoptosis following antigen clearance [1]. However, in LGL leukemia the LGLs persist. The WHO currently classifies T-cell large granular lymphocytic (T-LGL) leukemia as a subtype of mature peripheral T-cell neoplasms. It also separates T-cell leukemia and aggressive NK-cell leukemia into two different cancers. In 2008, a chronic form of NK-LGL was recognized as a provisional entity by the WHO, utilizing chronic NK-cell lymphocytosis to distinguish it from the more aggressive NK-LGL leukemia [2].

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*Author for correspondence: Tel.: +1 717 531 4034 ■ Fax: +1 717 531 0490 ■ tloughran@hmc.psu.edu.

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Current treatment & prognosis

The majority of patients are treated with low-dose immunosuppressive therapy [3]. Treatment is initiated in most cases due to severe neutropenia, anemia or rheumatoid arthritis (RA) [3]. However, the absence of large prospective trials contributes to the lack of a standard treatment regimen. It has been proposed that initial therapy should consist of low-dose methotrexate for patients with neutropenia. Methotrexate or cyclophosphamide is suggested for patients who are symptomatic with anemia. If treatment with methotrexate or cyclophosphamide is not successful, the patients are switched to the opposite agent or to cyclosporine. Most patients experience an indolent, chronic course and death infrequently occurs as a direct result of the disease [3].

The lack of a definitive cure or a targeted therapy places a great emphasis on establishing novel therapeutic targets. This review will investigate various dysregulated signaling pathways involved in LGL leukemia pathogenesis (Figure 1). The identification of novel therapeutics and current strategies for applying them will be reviewed.

LGL leukemia pathogenesis

Fas–Fas ligand signaling

Lymphocyte homeostasis is a balance maintained through lymphocyte proliferation and lymphocyte apoptosis. This balance is necessary for immune function. Dysfunction can lead to cancer and autoimmunity [1]. The activation and proliferation of antigen-specific naive T-cells is dependent on antigen recognition. Within a few days of antigen recognition there is an approximately 50,000-fold proliferation of antigen-specific T cells with the acquisition of T-cell effector functions. These activated T cells are eliminated upon antigen clearance through a process known as activation-induced cell death (AICD) [1]. This process is an important mechanism for the maintenance of self-antigen tolerance. The execution of AICD is attained in part through death receptor-mediated signaling and Fas–Fas ligand (FasL)-mediated apoptosis [4].

Upon CTL activation both Fas and FasL are upregulated to prepare these cells for effective elimination after antigen clearance. Fas, a member of the TNF receptor family, is involved in transducing death signals [4]. Upon binding of FasL with Fas, there is a subsequent trimerization of Fas. After trimerization, the adaptor protein FADD binds to the cytosolic portion of the receptor. This is collectively known as the death-inducing silencing complex (DISC) [5]. The DISC leads to binding and activation of procaspase-8. Procaspase-8 is activated proteolytically and the active form, caspase-8, is released into the cytoplasm. Activated caspase-8 cleaves a variety of downstream apoptotic effectors, including procaspase-3. This results in completion of death receptor-mediated apoptosis [5].

Dysregulation of Fas–FasL-mediated apoptosis in LGL leukemia

Similar to activated CTLs, leukemic LGLs from patients had constitutively high levels of Fas–FasL. However, LGLs from these patients were resistant to Fas–FasL-mediated apoptosis [6]. Molecular profiling of T-cell LGL leukemia shows a gene expression signature with dysregulation and uncoupling of activation and apoptotic pathways in AICD. The leukemic LGLs have acquired effector functions but many pro-apoptotic genes are downregulated and many antiapoptotic genes are upregulated (Figure 1) [7]. No known mutations in Fas or FasL have been found suggesting the resistance to Fas–FasL-mediated apoptosis is not inherent to Fas–FasL [8]. In addition, the inhibition of a variety of survival pathways leads to restoration of Fas–FasL-mediated apoptosis, suggesting the role of intact Fas–FasL apoptotic machinery [9].

In LGL leukemia patients there was an elevation of a soluble form of Fas (sFas) in their sera compared with healthy controls. It is believed that sFas competes with Fas by acting as a soluble decoy receptor and contributes to apoptotic resistance in leukemic LGLs. This was further supported by determining the source of sFas to be the LGLs themselves; the supernatant of COS cells that were transfected with sFas constructs cloned from LGLs was able to block Fas monoclonal antibody-induced apoptosis in leukemic LGLs [8].

Human FasL can also be converted to a soluble FasL (sFasL) through a matrix metalloproteinase (MMP)-like enzyme. Interestingly, sFasL was undetectable in sera from healthy controls, whereas LGL leukemia patients had high levels [10]. This provides an interesting target for therapeutic intervention.

Therapeutic interventions

While in principle the use of systemic Fas agonists or anti-Fas antibodies seems reasonable to induce apoptosis in leukemic LGLs, serious biological toxicity does not permit their use as therapeutics [11]. Homotrimeric sFasL is biologically inactive and is nontoxic to both normal and malignant cells. However, aggregated sFasL hexamers are highly tumoricidal but also demonstrate liver toxicity [12–14]. The targeting of sFasL to tumor cells through the use of tumor marker-specific antibodies has reduced systemic toxicity and has shown potential in various hematologic malignancies [15].

MMP inhibitors could hypothetically be used for the treatment of LGL leukemia. The blockage of MMP would prevent the production of sFasL from membrane-bound FasL, thus decreasing its levels in sera. There are a multitude of MMP inhibitors in clinical trials, such as marimastat [16]. Their potential use in LGL leukemia is an interesting hypothesis.

Ras–Raf-1–MEK1–ERK signaling

The *Ras* genes play an important role in tumor development. They represent one of the most common targets for gain-of-function mutations in cancer. These mutations occur in up to 30% of all cancers. The Ras proteins belong to a family of GTPases. The activation of Ras occurs in response to a multitude of extracellular signals including soluble growth factors. Downstream effector cascades play major roles in the survival, proliferation and differentiation of cells [17].

Ras signaling is central to the prosurvival regulation of T cells upon their activation on antigen recognition. The transmission of T-cell receptor (TCR) signals to Ras involves immunoreceptor tyrosine-based activation motifs [18]. Ras activity can further be modified through post-transcriptional modifications. Prenyl transferases, including farnesyltransferases and geranylgeranyl transferases, act to modify Ras activity by adding the hydrophobic moieties farnesyl or geranylgeranyl, respectively, to the C-terminus of Ras. This allows for the anchoring of Ras onto the cytosolic side of the cellular membrane, which is necessary for activation [17].

Once Ras signaling is initiated, Ras phosphorylates Raf-1 to activate it. Raf-1 phosphorylates and activates MEK1, which in turn phosphorylates MAPK. MAPK then acts downstream to phosphorylate ERK, which can subsequently activate a variety of downstream effectors or translocate to the nucleus. In the nucleus, ERK can phosphorylate transcription factors including Fos, Jun and CREB, among others. These transcription factors bind to the promoters of many genes involved in growth, proliferation and the prevention of apoptosis. Dysregulation can lead to abnormal cellular proliferation and leukemic transformation [19].

In activated Jurkat T cells and primary peripheral T cells, Ras–Raf-1–MEK1–ERK signaling acts to initially inhibit CD95/Fas-mediated apoptosis. The initial resistance of activated T cells to Fas-mediated apoptosis results from MAPK activity blocking the downstream effectors of DISC, but allowing DISC formation to occur as it normally would [20]. Essentially, the antiapoptotic MAPK pathway initially overrules the proapoptotic Fas-mediated signaling.

Dysregulated Ras–MEK1–ERK in LGL leukemia

In NK-LGL leukemia patients, the Ras–MEK1–ERK pathway has been found to be constitutively activated [21]. Inhibiting Ras, MEK1 or ERK induced apoptosis and restored Fas sensitivity in leukemic LGLs. The use of the MEK1 inhibitors PD98059 or U0216 reduced ERK activity and induced apoptosis. Furthermore, use of a dominant-negative form of MEK1 also induced apoptosis. The inhibition of Ras with the farnesyltransferase inhibitor FTI2153 or a dominant-negative form of Ras also induced apoptosis by inhibiting ERK activity. These results suggest that overactive Ras may play a role in the survival signaling in LGL leukemia.

Therapeutic interventions

In order for certain Ras isoforms to acquire malignant transforming activity they must first be farnesylated [22]. The targeting of Ras activity with farnesyltransferase inhibitors was therefore hypothesized to be a therapeutic option for LGL leukemia patients. The drug R115777 (also known as tipifarnib; Zarnestra®, Johnson & Johnson, New Brunswick, NJ, USA) is currently being investigated in clinical trials for various cancers, including leukemia [23]. However, a clinical trial with eight LGL leukemia patients using Zarnestra was conducted without any patients achieving clinical hematologic response [101].

In one patient with NK-LGL leukemia and concomitant primary pulmonary artery hypertension, farnesyltransferase inhibition with tipifarnib led to resolution of pulmonary artery hypertension symptoms [24]. It was proposed that pulmonary endothelial cells were being killed by the patient's NK cells, which became activated following dysregulation of activating and inhibitory NK receptor signaling pathways. The blockage of Ras signaling with tipifarnib may block the downstream signaling effectors (Ras–MEK1–ERK and PI3K–Akt) of NK receptor (or TCR in T-LGL leukemia).

PI3K–Akt signaling

The PI3K–Akt pathway has a central role in cancer progression by enhancing metabolism, proliferation, survival and the transformation of cells. The PI3Ks are activated by growth factors through receptor tyrosine kinases (RTKs) but can also be activated downstream of the Ras signaling cascade [25]. In human cancers, PI3K signaling is activated through mutational activation or amplification of PI3K- α or Akt1, or through a loss of phosphatase and tensin homolog, a negative regulator of the pathway. Furthermore, PI3K activity can be increased through aberrant activation of upstream RTKs or the Ras cascade [25].

In T cells, PI3K–Akt signaling is engaged through the Ras cascade or through interaction of the PI3K SH2-binding domain with cytokine receptors [26]. PI3K translocates to the membrane through interaction with Ras or Src family kinases (SFKs), or through direct interaction with cytokine receptors at SH2 domains. At the membrane, PI3K phosphorylates PIP₂ to produce PIP₃, which then activates the most-studied downstream effector of PI3K signaling, Akt (or PKB). Activated Akt promotes proliferation through stimulating mTOR and by upregulating cyclin D1 [25].

Activated Akt also acts in prosurvival signaling by blocking the inhibition of the NF- κ B transcription factor, leading to increased transcription of prosurvival and antiapoptotic genes [27]. In addition, Akt is antiapoptotic through inhibition of both the proapoptotic Bcl-2 family member Bcl-2 antagonist of cell death and procaspase-9 through phosphorylation [25]. For example, the phosphorylation of Mdm2 by Akt enhances its ability to bind and inhibit the tumor suppressor p53 [27].

Dysregulated PI3K–Akt in LGL leukemia

In T-LGL leukemia, PI3K–Akt pathway activity was found to be increased in T-LGL cells compared with T cells from healthy donors (Figure 1) [28]. This activation was SFK dependent and PI3K was kept in a constitutively active form, evaluated through the phosphorylated state of Akt and GSK-3. In addition to SFK, defects in the negative regulation of this pathway were found. Decreased expression of PAG1, a negative regulator of SFK, and phosphatase and tensin homolog, a negative regulator of PI3K–Akt activation, has been found in T-LGL cells [28]. Inhibition of SFK- or PI3K-induced apoptosis, in addition to decreasing ERK1/2 expression, suggests a connection between PI3K–Akt activity and the Ras–MEK1–ERK pathway [29].

Therapeutic interventions

Therapeutics targeting PI3K–Akt signaling are actively being studied at the preclinical and clinical levels in a variety of advanced solid tumors. The major role of this pathway in cell proliferation and survival makes it an excellent candidate for anticancer therapy. PI3K inhibitors can be divided into pan-PI3K inhibitors, which target all isoforms, and isoform-specific inhibitors. The role of the PI3K–Akt cascade is widespread and pan-PI3K inhibitors have been shown to have substantial side-effect profiles. Pan-PI3K inhibitors such as PX-866 and GDC-0941 are all in Phase I trials for solid tumors and show promise, but patients experience a wide range of side effects [30,31]. Since the p110 α catalytic subunit of PI3K is often mutated in cancer, it would be ideal to target this isoform specifically [32]. The PI3K–Akt pathway has also been shown to be dysregulated in various autoimmune diseases [33]. Given the association of LGL leukemias with RA and other autoimmune disorders it would be interesting to investigate PI3K inhibitor compounds in LGL leukemia patients.

JAK–STAT signaling

STAT proteins are latent transcription factors that mediate a wide variety of biological processes such as cell proliferation, apoptosis, angiogenesis and immune responses (Figure 2). The STAT family of proteins consists of at least seven members: STAT1–4, STAT5a, STAT5b and STAT6, which are encoded by different genes [34]. Structurally, these proteins share a similar organization, including an N-terminal domain, a coiled-coil region, a DNA-binding domain, an SH2 domain and a C-terminal trans-activation domain. In signal transduction, STAT proteins serve as the converging point of three signaling pathways that are mediated through cytokine receptors, growth factor receptors (tyrosine kinases) and nonmembrane-bound tyrosine kinases, respectively [34–36]. A typical signaling event begins with the engagement of cytokines (e.g., IFN- α or IL-6) or growth factors (e.g., EGF or PDGF) with their receptors, which leads to autophosphorylation of the receptor-associated tyrosine kinases that are members of JAKs and SFKs [37,38]. These kinases in turn phosphorylate the cytoplasmic tails of the cytokine receptors on specific tyrosine residues. The STAT monomers in the cytoplasm are then recruited through the SH2 domain and are subsequently phosphorylated by the JAKs or SFKs, resulting in dimerization of STAT monomers through reciprocal SH2 domain interactions [34,39]. The newly formed

STAT homo- or hetero-dimers then rapidly translocate to the nucleus where they exert their transcriptional activities by binding to STAT response regions of the target genes.

Dysregulation of the JAK–STAT3 pathway in LGL leukemia

In normal cells, the duration of activation of individual STAT proteins is tightly regulated, lasting from a few minutes to several hours. However, aberrant activation of JAKs or other types of tyrosine kinases can lead to persistent activation of STAT proteins, especially STAT3, which has been found in more than 22 types of cancer cell lines and primary cancers, and is linked to enhanced cell proliferation, survival and malignant transformation [40].

When compared with the studies in other cancers, the knowledge of JAK–STAT signaling in leukemic LGLs is still limited. STAT3 has been found to be constitutively activated in patients with leukemic LGLs [9]. Recent sequencing analyses of leukemic CTLs have revealed that 40% (31/77) of LGL leukemia patients carry somatic mutations in the SH2 domain of STAT3, a critical region that mediates the dimerization and activation of STAT3 [41]. Interestingly, the LGL leukemia patients with these STAT3 mutations presented more often with neutropenia and RA than those without the mutations. Furthermore, several STAT3 target genes (*BCL2L1*, *INFGR2* and *JAK2*) were found to be upregulated in leukemic LGL patients with STAT3 mutations in the SH2 domain [41]. This effect can also be conferred by *in vitro* expression of the STAT3 SH2 mutants, which resulted in an increase in the transcriptional activity of the STAT3 protein [41]. On the other hand, blockade of STAT3 using STAT3 antisense oligonucleotide induced apoptosis as well as restored Fas sensitivity in leukemic LGLs [9]. Collectively, aberrant JAK–STAT3 signaling underlines the pathogenesis of LGL leukemia and STAT3 represents a desirable therapeutic target in LGL leukemia patients.

Therapeutic interventions

STAT3 stands at the point where all the three signaling pathways converge; therefore, it represents a promising target. Strategies can be diverse, targeting the phosphorylation, dimerization, nuclear translocation and DNA-binding ability of STAT3. Several compounds that are designed to target the DNA-binding activity of STATs have shown antitumor activity. For example, a small peptide (P[pTyr]LKTK), which targets the SH2 domain of STAT3, has been shown to block STAT3 DNA-binding activity and STAT3 dimerization *in vitro* [42]. Subsequently, a peptidomimetic analog (ISS610) and an oxazole, S31-M2001, have been developed. *In vitro* application of ISS610 led to the blockade of constitutive STAT3 activity, growth inhibition and induction of apoptosis of the human breast carcinoma cells [43,44]. S31-M2001 exhibits similar intracellular activity but at a lower concentration compared with ISS610 [43]. It will be of interest to determine the intracellular activity and anti-tumor effects of these drugs in cancer models when the limitations (such as low cell permeability and low stability) are overcome. Of note, high rates of mutations were identified within the STAT3 SH2 domain in LGL leukemia patients [41], thus small peptides targeting the mutant region of STAT3 might represent another novel therapeutic target.

Recently developed small molecules that target STAT3 can be of use in targeting various cancers [45]. OPB-31121 is a novel STAT3 inhibitor that selectively inhibits IL-6-dependent phosphorylation of STAT3 without inhibiting JAK2 phosphorylation [45]. OPB-31121 demonstrated strong growth suppression in a wide range of cancer cell lines, especially cells from hematopoietic malignancies including acute myeloid leukemia, chronic myeloid leukemia and myeloma [45]. Further studies showed that OPB-31121 significantly suppressed human leukemic cell growth in mouse models. Currently, Phase I clinical trials

of OPB-31121 for advanced solid tumors and Phase I/II trials for progressive hepatocellular carcinoma are ongoing [102,103]. In addition, a small molecule, C48, was found to selectively block DNA binding of STAT3 by alkylating Cys468 in STAT3, a residue at the DNA-binding interface. This blocked accumulation of activated STAT3 in the nucleus, leading to the inhibition of tumor growth in this mouse model [46]. This suggests that Cys468 in STAT3 represents a novel site for therapeutic intervention [46].

STAT3 decoy is a synthetic double-stranded oligodeoxynucleotide, which mimics the consensus sequence of the *cis*-element of STAT3-targeted genes, and has a highly specific affinity to the transcription factor. *In vitro* and *in vivo* studies showed that decoy oligodeoxynucleotide blocked the STAT3–*cis*-element interaction, resulting in the downregulation of the STAT3-targeted genes (*cyclin D*, *survivin*, *Bcl-X_L*) [47]. Application of the STAT3 decoy led to reduced proliferation and induction of apoptosis in a variety of human tumor cell lines and animal models [48,49]. The Phase 0 clinical trial of STAT3 decoy for head and neck cancers was completed recently [104].

Besides inhibition of STAT proteins, targeting upstream tyrosine kinases (JAKs) is another rational strategy for anticancer therapy. CYT387 is a novel JAK inhibitor that can inhibit JAK1–3 and nonreceptor TYK2 [48,49]. Preclinical experiments demonstrated that CYT387 blocked IL-6-induced phosphorylation of STAT3 in multiple myeloma cell lines. Further study showed that CYT387 inhibited proliferation and disrupted cell cycle in human multiple myeloma cells [50]. CYT387 is currently under evaluation in Phase II clinical trials for primary myelofibrosis [105].

Dysregulation of sphingolipid rheostat in LGL leukemia

Sphingolipids, a class of lipids containing a backbone of sphingoid bases, are key structural components of biological membranes. Sphingolipid metabolites control diverse cellular processes such as proliferation, apoptosis and migration [50]. *De novo* sphingolipid synthesis begins in the endoplasmic reticulum with the formation of 3-keto-dihydrosphingosine from serine and palmitate [50]. This product then undergoes reduction, acylation and desaturation to give rise to ceramide, a fundamental structural unit common to all sphingolipids. Ceramide, being the backbone of sphingolipid molecules, is central to sphingolipid metabolism and has several fates. Further modifications (e.g., phosphorylation and glycosylation) in the Golgi network give rise to complex sphingolipids that are subsequently transported to the plasma membrane. Alternatively, this molecule can be broken down into diverse bioactive products. It can be phosphorylated by CK to form ceramide 1-phosphate (C1P), or deacylated by ceramidase to generate sphingosine, which can be further phosphorylated by SphK1 or SphK2 to yield sphingosine 1-phosphate (S1P) (Figure 3). In addition, it can also be converted into sphingomyelin by sphingomyelin synthase. In general, these steps are reversible and can be converted by corresponding enzymes. Consequently, there exists a balance among these metabolites.

These sphingolipid metabolites play important but opposite roles in biological processes. Ceramide and sphingosine are proapoptotic factors, inducing cell growth arrest and apoptosis. By contrast, S1P promotes cell survival and growth by acting either intracellularly or extracellularly through the S1P receptors, GPCRs, which consist of five isoforms (S1PR1–S1PR5) [51,52]. The balance between proapoptotic (such as ceramide and sphingosine) and prosurvival (such as S1P and C1P) sphingolipids is believed to provide a ‘sphingolipid rheostat’ mechanism that eventually determines the fate of the cell (Figure 3) [50,53].

Considering the critical functions of sphingolipids in regulating cell proliferation and apoptosis, it is not surprising to see that the sphingolipid rheostat is dysregulated in a way

that tilts the balance in favor of survival molecules (e.g., S1P) and away from proapoptotic molecules (such as ceramide) in a variety of human cancers [50]. Studies from the authors' laboratory, as well as others, have provided convincing evidence that the balance is dysregulated in the cells of LGL leukemia patients; a few examples are given below.

First, ASAHI, which regulates the rate-limiting step in conversion of ceramide into sphingosine, was constitutively overexpressed, resulting in low levels of ceramide and survival of leukemic LGL cells [7]. Pharmacological inhibition of this enzyme by specific chemical inhibitor NOE led to significant apoptosis of leukemic LGLs *in vitro* [7]. The second example is SphK1, a kinase that converts the death-promoting sphingosine to the growth-promoting S1P. SphK1 is upregulated in leukemic LGLs and a variety of other tumors [52,54,55]. SphKs crosstalk with several survival signaling pathways, such as Ras–MEK1–ERK, NF- κ B and PI3K–Akt, which constitute a positive-feedback loop [55,56]. Elevated activity of SphKs increases the concentration of S1P while reducing the levels of ceramide and sphingosine. Overproduction of S1P has been shown to contribute to cancer cell survival, proliferation and migration [55].

In addition to the alteration of the sphingolipid metabolic pathway, there have been changes in the expression level of the sphingosine receptors. In normal human CD8⁺ naïve T cells, S1PR1 is the predominant type on the cell surface while other isoforms are expressed at low levels. By contrast, in leukemic LGLs, S1PR5 is constitutively overexpressed and is the predominant S1P receptor [54]. Hypothetically, the G-protein signaling mediated by G α_{12} coupling with S1PR5 is upregulated in leukemic LGLs, which activates the prosurvival Ras–MEK1–ERK signaling that is constitutively activated in LGL leukemia, promoting cell survival, invasion and metastasis [7,21].

Collectively, the sphingolipid rheostat is deregulated in LGL leukemia; therefore, modulation of the balance in this rheostat by increasing the levels of ceramide and decreasing activities or levels of ASAHI, SphKs, S1P and S1PR5 might serve to drive successful therapies in LGL leukemia.

Therapeutic interventions

The proapoptotic metabolites (ceramide and sphingosine) promote apoptosis and exhibit antiproliferative and proapoptotic activity in many cancer cells [57]. However, their potential therapeutic benefits as cancer chemotherapy are limited due to their hydrophobicity and the obstacles to delivering it for systemic application. Recent development of nanoparticles has provided a promising means for drug delivery. It has been reported that a pegylated nanoliposomal formulation of cell-permeable C₆-ceramide inhibits tumor growth in mouse models of human breast adenocarcinoma and melanoma [58,59]. In addition, studies from the authors' laboratory showed that nanoliposomal ceramide induces complete remission in a rat model of NK-LGL leukemia, a fatal illness with no known curative therapy [60]. Further investigation demonstrated that C₆-ceramide down-regulates the expression of survivin (an inhibitor of apoptosis protein) by inhibiting ERK, an essential molecule of the survival pathway in LGL leukemia and a variety of other tumors. Moreover, the nanoliposomal C₆-ceramide was found to induce cell death in leukemic NK cells in a caspase-dependent manner [60]. Finally, as higher leukemic burden is a contributing factor to the inability to obtain complete remission, improvement in therapeutic efficacy might be achieved by developing immuno-nanoliposome C₆-ceramide targeting a specific type of cells, such as CD8- and CD56-expressing cells in NK leukemia, and CD3- and CD8-expressing cells in T-LGL leukemia.

The second approach is to inhibit the activity of the antiapoptotic metabolites (S1P and C1P) by antibodies. Currently, S1P-neutralizing antibodies are in development as new

therapeutics. A monoclonal antibody against S1P (anti-S1P mAb) was shown to have very impressive efficacy even though this antibody was found binding to not only S1P, but also dihydro-S1P and sphingosylphosphorylcholine [61]. Interestingly, LT1009 (ASONEP™, Lpath Inc., San Diego, CA, USA), an S1P-specific antibody, was shown to deplete S1P from the blood and is currently in Phase I trials for cancer and age-related macular degeneration [106].

An alternative approach targeting sphingolipid metabolism is to synthesize functional antagonists of antiapoptotic metabolites. FTY720, a prodrug that was approved by the US FDA as an immunomodulatory drug for the treatment of multiple sclerosis, represents such a compound [62]. FTY720 is structurally similar to S1P, phosphorylated *in vivo* by SphK2 and binds to four of five S1P receptors (except S1PR2). Accumulated evidence has shown that FTY720 induces apoptosis and reduces tumor growth in many human cancer cells and cancer models, including breast cancer, chronic lymphocytic leukemia and Philadelphia chromosome-positive acute lymphocytic leukemia [63–65]. Recently, the authors reported that systemic administration of FTY720 induced complete remission in a rat model of NK-cell leukemia. FTY720 induces apoptosis of leukemic NK cells by promoting the degradation of survival signaling component Mcl-1 and increasing the proapoptosis lipid sphingosine [66]. Given that C₆-ceramide and FTY720 act on distinct cellular targets in NK-cell leukemia, it will be interesting to test their therapeutic efficacy in combination treatment in LGL leukemia.

As discussed above, S1PR5 has been shown to be predominantly upregulated in LGL leukemia; however, no specific agonist or antagonist for S1PR5 is reported even though agonists for S1PR1 (such as KRP-203 and SEW2871), antagonists for S1PR1/3 (VPC23019) and antagonists for S1PR3/5 (suramin) have been discovered recently [67]. Therefore, screening for an S1PR5 agonist and antagonist is an urgent need for the development of therapeutics in LGL leukemia. In addition, the key regulators of the sphingolipid rheostat, the SphKs that generate S1P and decrease sphingosine and ceramide, are promising targets to treat cancer, including LGL leukemia.

NF-κB signaling

NF-κB is a transcription factor complex and has proven roles in hematopoiesis, inflammation and functioning and survival of immune cells. Originally discovered through the research area of immunology, NF-κB now has a well-established role in cancer biology [68]. In an inactivated state, NF-κB is found in the cytoplasm, complexed with a member of the inhibitor of NF-κB (IκB) family, preventing its translocation into the nucleus and denying its role as a transcription factor. Once the NF-κB pathway is activated, an IκB kinase (IKK) phosphorylates IκB, thus initiating its subsequent ubiquitination and proteosomal degradation. IKK is downstream of a multitude of pathways including PI3K–Akt as a substrate for AKT (Figure 1) [69].

Upon TCR activation in T cells, NF-κB is relied upon for protection against apoptosis and the production of prosurvival proliferative cytokines. This activation of NF-κB downstream of TCR permits T cells to acquire effector functions and the ability to produce IL-2, a major cytokine necessary for the growth and function of T cells. Inhibition or loss of NF-κB leads to AICD and apoptosis [68]. NF-κB inhibits AICD through promoting the expression of prosurvival Bcl-2 family members (including Mcl-1).

Dysregulated NF-κB in LGL leukemia

The importance of NF-κB signaling to T-cell activation and survival prompted investigation into its role in LGL leukemia. Gene expression data of LGL leukemia showed that c-Rel, a

member of the NF- κ B family, was over-expressed [7]. Furthermore, a network model of survival in T-LGL leukemia demonstrated that NF- κ B is constitutively active in T-LGLs [70]. In nuclear extracts of T-LGLs, NF- κ B exhibited high levels of activity versus normal PBMCs, in which activity was rarely exhibited. Inhibition of NF- κ B in T-LGLs with the specific inhibitor BAY 11-7082 led to apoptosis of these cells. The network model also showed that NF- κ B activity was dependent on the state of the PI3K signaling pathway. When T-LGL cells were given the PI3K inhibitor LY294002, apoptosis was induced and NF- κ B activity inhibited [70]. However, the inhibition of NF- κ B did not affect the phosphorylation status of Akt, suggesting that NF- κ B is downstream of PI3K. It was shown that NF- κ B prevents apoptosis through Mcl-1, independently of STAT3 [70].

Therapeutic interventions

The central role of NF- κ B in proliferation, survival and immune function make it an ideal candidate for therapeutic targeting in leukemia. Unfortunately, the diverse role it plays in normal cellular functions provides substantial risk for widespread toxicity to patients.

Bortezomib is a protease inhibitor that prevents the degradation of I κ B. The accumulation of I κ B in cells treated with bortezomib leads to the suppression and downregulation of targets downstream of NF- κ B signaling and the apoptosis of cells [71]. In 2003, bortezomib was approved by the FDA for treating relapsed multiple myeloma and mantle cell lymphoma based on Phase II trial results [72]. Preclinical studies of bortezomib in T-LGL leukemia are promising. The cells exhibit a dose- and time-dependent apoptotic response to the addition of bortezomib [73].

IL-15 signaling

IL-15 plays an important role in the regulation and survival of T and NK cells. In order for memory cells to persist following antigen clearance, cytokines such as IL-2 and IL-15 must be present. IL-2 and IL-15 are similar in that their receptors are heterotrimeric and contain two identical subunits with one unique subunit: IL-12/15R β . In view of their common receptors and signaling pathways, IL-2 and IL-15 were thought to play similar roles in immune system function. This is true in that they both generate CTLs and generate and maintain NK cells [74]. However, in CTL homeostasis, IL-2 and IL-15 have opposing roles. IL-2 is involved in the elimination of self-reactive T cells through AICD and helps prevent the development of autoimmune diseases. By contrast, IL-15 is important for supporting the survival and proliferation of CD8⁺ memory T cells [74]. IL-15 also supports the survival of NK cells.

As mentioned above, a network model of survival signaling was developed for T-LGL leukemia. The network was created by integrating known signaling pathways of CTL activation and the known dysregulations of survival signaling in T-LGL leukemia. A predictive discrete dynamic Boolean model was applied and simulating node dysregulations corresponding to known signaling dysregulations were employed. The model predicted that the constitutive presence of IL-15, activation of PDGF signaling and the initial T-cell activation are sufficient to reproduce all dysfunction present in the signaling pathways of leukemic T-LGLs. These findings were verified experimentally, validating the results of the network model [7].

Based on the involvement of IL-2 and IL-15 in the survival of both CTL and NK cells, it is not surprising that IL-15 plays an important role in the pathogenesis of T- and NK-LGL leukemia. The inhibition of IL-15 leads to apoptosis in leukemic LGL cells. This inhibition also increases the levels of Bid, a proapoptotic protein that was found to be decreased in leukemic T- and NK-LGL cells from patients when compared with normal controls [73].

This suggests that Bid plays a role in the abnormal signaling in leukemic LGLs downstream of IL-15. Overexpressing Bid through transduction in T-LGL leukemia cells led to enhanced apoptosis.

Bid is a member of the BH3-only group of the Bcl-2 family. It is a substrate for caspase-3 and caspase-8 in the DISC signaling pathway, as well as for granzyme B. After Bid is cleaved, it translocates to the outer mitochondrial membrane, acting to promote caspase-induced death signaling. To terminate the signal, an E3 ubiquitin ligase, HDM2, targets Bid to the proteasome for degradation. In normal NK cells, IL-15 has been shown to upregulate HDM2 and target Bid to the proteasome [73].

The soluble IL-15R α receptor has been shown to be upregulated in the serum of patients with T-LGL leukemia [75]. IL-15R α mRNA was also upregulated in the PBMCs of these patients. Interestingly, PBMCs of leukemic T-LGL patients responded to IL-15 stimulation with more proliferation than normal donors. It was suggested that increased IL-15R α may lower the threshold for IL-15 stimulation in leukemic T-LGL, contributing to the pathogenesis of the disease [75].

Therapeutic interventions

As mentioned above, bortezomib is a proteasomal inhibitor that has shown efficacy in a variety of hematologic cancers. In leukemic LGLs, in addition to its effects on the NF- κ B pathway, bortezomib treatment of leukemic T- or NK-LGL led to the induction of apoptosis with accumulation of Bid, suggesting a role for Bid outside of NF- κ B and independent of Fas or TRAIL-induced apoptosis [73].

Preclinical and Phase I clinical trials using murine Mik β 1, a monoclonal antibody to CD122 (β -subunit shared by IL-2 and IL-15 receptors), demonstrated no significant toxicity or immune response to the antibody. There was no reduction in peripheral leukemic LGL count in any of the 12 patients tested but they did demonstrate downregulation of the receptor on the cell surface [76]. The lack of immunogenicity and toxicity with Mik β 1 in these patients suggests that a targeted approach using molecular conjugation of other therapeutics to Mik β 1 may be a viable option [15].

PDGF signaling

PDGF is one of the major growth factors that regulates cell growth and division. The PDGF family is composed of five different isoforms, A, B, C and D and an AB heterodimer. A molecule of PDGF is dimeric and composed of homodimeric proteins (PDGF-AA, PDGF-BB, PDGF-CC or PDGF-DD) or heterodimeric PDGF-AB protein. PDGF signaling acts through three receptor complexes containing two subunits, PDGF- α or PDGF- β . Three different receptor complexes exist as PDGF- $\alpha\alpha$, PDGF- $\beta\beta$ or PDGF- $\alpha\beta$. The PDGF- α and PDGF- β subunits are type III RTKs and act downstream through many of the major signaling pathways mentioned previously, such as JAK-STAT, PI3K-Akt and Ras-MEK1-ERK [77]. It is no surprise then that PDGF signaling results in cell proliferation and growth. The initial role of PDGF in cancer was suggested after *v-sis* (the oncogene of simian sarcoma virus) was found to be 92% homologous to PDGF- β [78]. Subsequent studies proved that deregulated PDGF signaling was associated with a variety of malignancies including leukemia [77].

Dysregulated PDGF signaling in LGL leukemia

As illustrated above, the constitutive presence of IL-15 and activation of PDGF signaling was sufficient after T-cell activation to produce all known dysregulations in T-LGL leukemia [70]. Like IL-15, PDGF can be considered central to the pathogenesis of leukemic

T-LGLs. The network model was verified experimentally with PDGF-BB being elevated in T-LGL leukemia patient sera compared with control sera [79]. Production of PDGF-BB was traced to T-LGLs through immunocytochemical staining. In addition, leukemic LGLs expressed higher levels of PDGFR- β when compared with normal CD8⁺ controls [79].

The downstream effector pathways of PDGF in leukemic LGLs are the PI3K–Akt and MEK1–ERK signaling cascades. Pharmacologic blockade of PI3K activity (with LY294002) resulted in apoptosis of both T- and NK-leukemic LGLs and prevented PDGF-BB-induced activation of Akt and ERK, suggesting that PI3K activation acts as a gatekeeper between PDGF action and the downstream Akt and ERK pathways [79]. To confirm, a neutralizing antibody to PDGF-BB inhibited Akt phosphorylation and induced apoptosis in leukemic LGLs [79]. Finally, the chemical inhibitor AG1296 was employed to block the RTK activity of PDGF- β . This also resulted in apoptosis of leukemic LGL [70,79].

Therapeutic interventions

The prominent role of PDGF signaling upstream of many of the dysregulations in LGL leukemia identify it as an ideal therapeutic target. Imatinib mesylate (also known as STI-571; Gleevec®, Novartis, East Hanover, NJ, USA) is a well-studied RTK inhibitor that acts on Abl, c-KIT, PDGF receptor and notably the Bcr-Abl oncoprotein found in chronic myeloid leukemia [80]. The antitumor role of imatinib is well established but recently it has also been suggested as a therapeutic for autoimmune disorders such as RA [80].

Conclusion & future perspective

LGL leukemia is a disease of clonal expansion of CTLs. There are no curative therapeutic modalities for LGL leukemia; therefore, new treatment options are needed. Importantly, LGL leukemia seems to result from dysregulation of multiple survival pathways.

Investigation into the dysregulated survival pathways of LGL leukemia will allow researchers to identify, and screen for, potential targeted therapeutics for this incurable disease. The role of basic scientific research into the pathogenesis of LGL leukemia is vital in order to elucidate the connections between the multiple dysregulated pathways that appear to be central to LGL survival. New targeted therapies that are currently being studied offer promising opportunities for LGL research due to recognition of the important Fas–FasL, sphingolipid, Ras–MEK1–ERK, PI3K–Akt, JAK–STAT3, NF- κ B, IL-15 and PDGF pathways in leukemia survival.

The recognition of potential therapeutics for LGLs based on the above pathways will allow screening in *in vitro* cell models and *in vivo* animal models, hopefully with the transition into human clinical trials. Overall, within the next 5–10 years, targeted combination therapies for LGL leukemia should become evident.

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Executive summary

Current treatment & prognosis

- Current treatment for large granular lymphocyte (LGL) leukemia involves a low-dose immunosuppressive therapy.
- An absence of large prospective clinical trials contributes to a lack of standard treatment regimen and development of targeted therapies is therefore necessary.

Fas–Fas ligand signaling

- Leukemic LGL patients have constitutively high levels of Fas–Fas ligand (FasL) and elevated soluble Fas in their sera, but they are resistant to Fas–FasL-mediated apoptosis.
- The use of soluble FasL with a targeted tumor-specific antibody is a potential therapeutic that warrants investigation.

Ras–MEK1–ERK signaling

- Ras signaling is a major prosurvival pathway in the regulation of T cells.
- In natural killer (NK)-LGL leukemia patients the Ras–MEK1–ERK pathway is constitutively activated.
- The use of Ras, MEK1 or ERK inhibitors induced apoptosis and restored Fas–FasL-mediated apoptosis.
- A small clinical trial with R115777 (also known as tipifarnib; Zarnestra®, Johnson & Johnson, New Brunswick, NJ, USA) in LGL leukemia patients did not show clinical hematologic response.

PI3K–Akt signaling

- PI3K–Akt signaling is activated in T cells through the Ras cascade or interaction of the PI3K SH2-binding domain with cytokine receptors.
- In T-cell large granular lymphocytic (T-LGL) leukemia there is increased PI3K–Akt pathway activity.
- Inhibition of PI3K-induced apoptosis and therapeutics targeting this pathway are therefore excellent candidates for anticancer therapy.

IL-15 signaling

- A network model of survival signaling in T-LGL leukemia showed that constitutive presence of IL-15 and activation of PDGF signaling are sufficient to reproduce signaling pathway dysfunction.
- The soluble IL-15R α receptor is upregulated in the sera of patients with T-LGL leukemia.
- A targeted approach with Mik β 1, a monoclonal antibody to CD122, is possibly a viable future treatment option.

JAK–STAT signaling

- Recent sequencing analysis in LGL patients has revealed somatic mutations in the SH2 domain of STAT3 in 40% of patients.
- Blockade of STAT3 signaling induced apoptosis and restored Fas sensitivity in leukemic LGLs.

- STAT3 represents a promising target for therapeutic intervention through various strategies including phosphorylation, dimerization, nuclear translocation and DNA binding.

Dysregulation of the sphingolipid rheostat in LGL leukemia

- The balance between ceramide, sphingosine and sphingosine 1-phosphate influences proapoptotic and prosurvival pathways that determine cell fate.
- S1PR5 is constitutively overexpressed in leukemic LGLs.
- Nanoliposome formulations of C₆-ceramide have successfully been used to induce complete remission in a rat model of NK-LGL leukemia.

NF-κB signaling

- NF-κB is central for protection against apoptosis in activated T cells and NF-κB is constitutively activated in T-LGLs.
- Inhibition of NF-κB with the inhibitor BAY 11-7082 in T-LGL cells led to apoptosis.
- Bortezomib, a protease inhibitor, has shown promise in preclinical studies in T-LGL leukemia cells.

PDGF signaling

- PDGF activation, in addition to IL-15, is sufficient to reproduce all known deregulations in T-LGL leukemia.
- The network model of PDGF was verified experimentally with elevated levels of PDGF-BB in patient sera.
- Blockade of downstream signaling cascades of PDGF (PI3K–Akt and MEK1–ERK) resulted in apoptosis of both T-cell and NK leukemic LGLs.

Figure 1. Dysregulated survival pathways in large granular lymphocyte leukemia

The intact Fas–FasL-mediated apoptosis pathway is shown. Elevated levels of the soluble form of Fas or FasL contribute to apoptotic resistance in leukemic LGLs by acting as receptor or ligand decoys. MMP inhibitors are proposed for therapeutic intervention. Other contributing signaling pathways that are essential in LGL leukemia pathogenesis include the signals that are mediated by IL-15, PDGF and NF- κ B. IL-15 promotes LGL survival either by activating the JAK–STAT3 signaling pathway to upregulate gene expression of antiapoptotic factor or by targeting Bid to the proteasome for degradation. IL-15 signaling can be inhibited by a Mik β 1 monoclonal antibody targeting the IL-15R. PDGF functions through PI3K–Akt and Ras–MEK1–ERK signaling cascades, leading to cell proliferation and growth. PDGF signaling can be inhibited by RTK inhibitors such as imatinib. PI3K–Akt signaling can be blocked by using pan-PI3K inhibitors. Ras–MEK1–ERK signaling can be targeted using farnesyltransferase inhibitors to prevent farnesylation of Ras or by using MEK1 inhibitors such as PD98059 and U0216. NF- κ B complex upregulates the transcription of survival genes resulting in survival of leukemic LGLs. Bortezomib, a proteasome inhibitor, can be used to prevent NF- κ B signaling. FasL: Fas ligand; I κ B: Inhibitor of NF- κ B; IKK: I κ B kinase; IL-15R: IL-15 receptor; LGL: Large granular lymphocyte; MMP: Matrix metalloproteinase; RTK: Receptor tyrosine kinase.

Figure 2. The STAT signaling pathway

STATs serve as the converging point for many signaling pathways that are mediated through cytokine receptors, growth factor receptors and nonreceptor tyrosine kinases, which are commonly activated in cancer cells. First, stimulation of cells with growth factors or cytokines results in the activation of intrinsic receptor tyrosine kinase activity or of receptor-associated kinases. These kinases in turn phosphorylate the receptor cytoplasmic tail to provide docking sites for STAT monomers via their SH2 domain, resulting in phosphorylation of STATs by JAKs or Src kinases. Oncogenic Src and Abl can also phosphorylate STATs in a receptor-independent manner. Second, the phosphorylated STAT monomers dimerize and translocate to the nucleus. Finally, STATs bind to specific STAT DNA-response elements and directly regulate the expression of a variety of gene expression that are critical for cell proliferation (e.g., cyclin D1 and cyclin D2), survival (e.g., survivin), angiogenesis (e.g., VEGF) and immune evasion (e.g., immune-suppressing factor). In normal cells, STAT activation is tightly regulated; however, constitutive activation of STATs, in particular STAT3, is associated with human cancer development. Consequently, STAT3 emerges as an attractive target for cancer therapy. Proposed therapeutic interventions include JAK inhibitors (e.g., CYT387), preventing STAT3 dimerization (e.g., OPB-31121) and preventing STAT3 nuclear translocation (e.g., P(pTyr)LKTK and C48).

Figure 3. Dysregulation of sphingolipid metabolism and signaling in leukemic large granular lymphocytes

Decreased antiproliferative and apoptotic activities mediated by ceramide and sphingosine are illustrated. The upregulated enzyme activities or gene function in leukemic large granular lymphocytes are shown. Inhibition of ASAHI can be accomplished using NOE. S1P signaling can be blocked with fingolomid (FTY720). C₆-ceramide nanoliposome is an effective method of delivering ceramide into a cell. C1PP: Ceramide 1-phosphate phosphatase; S1P: Sphingosine 1-phosphate; S1PP: Sphingosine 1-phosphate phosphatase; S1PR: Sphingosine 1-phosphate receptor.