Large Granular Lymphocyte Leukemia – From Molecular Pathogenesis to Targeted Therapy

Mithun Vinod Shah and Thomas P. Loughran Jr.

Introduction

Large Granular Lymphocytes (LGL) are part of the immune system normally comprising 10–15% of adult peripheral blood mononuclear cells (PBMC). LGL actively survey for virus-infected or transformed cells in the body. Phenotypically, LGL contain two distinct subpopulations – cytotoxic T-lymphocytes (CTL) and Natural Killer (NK) cells. Despite important differences in origin and functions, CTL share several characteristics with NK-cells (Smyth et al. 2001).

LGL leukemia is a chronic lymphoproliferative disorder of cytotoxic lymphocytes. LGL leukemia can be of cytotoxic T-cells (known as T-cell LGL leukemia or T-LGL leukemia) or that of NK cells (NK-cell LGL leukemia or NK-LGL leukemia). In T-LGL leukemia, the expansion of clonal CTL is seen as the expansion of CD3+ CD8+/CD57+ T-cell receptor (TCR)-αβ+CTL (Loughran 1993). Leukemic T-LGL are CD45RA+CD62L-, a phenotype consistent with effector-memory RA T-cells (T_{EMRA}). Thus, leukemic T-LGL can be considered as malignant expansion of T_{EMRA} cells (Loughran 1993; Yang et al. 2008).

NK-LGL leukemia is characterized by CD3- CD56+ and/or CD16+ cells (Loughran 1993). In the absence of clonal TCR gene it is more difficult to establish the clonality of leukemic NK-LGL. However, recent reports studying NK-receptor

M.V. Shah
Department of Internal Medicine, Penn State Milton S. Hershey Medical Center,
500 University Drive, Hershey, PA, USA

T.P. Loughran Jr. (✉)
Department of Internal Medicine, Penn State Milton S. Hershey Medical Center,
500 University Drive, Hershey, PA, USA

Penn State Hershey Cancer Institute, Penn State Milton S. Hershey Medical Center,
500 University Drive, Hershey, PA, USA
e-mail: tloughran@psu.edu

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repertoire suggest clonal expansion of NK cells in NK-LGL leukemia patients (Loughran 1993; Epling-Burnette et al. 2004a).

Following recognition of target cells, LGL are powerful executers of cell-mediated cytotoxicity. Upon finding their targets, LGLs make brief contact with target cells and induce apoptosis in the latter. Apoptosis induction in the target cells is achieved either by death receptor mediated pathway or by delivering cytotoxins in the target cells. The death receptor mediated signaling depends on the activation of 'death receptors' such as Fas by their ligands. LGL contain various cytotoxins such as perforin (pore forming protein) and granzyme B (GrB) in their azurophilic granules. Perforin punches pores in target cells facilitating the delivery of cytotoxins such as GrB into the target cells. Once in target cells, GrB cleaves and activates various proteins such as caspases, filamin, nuclear poly(ADP-ribose) polymerase (PARP) and Bid leading to cell death in both caspase-dependent and -independent manners (Smyth et al. 2001; Krammer 2000).

Clinical Features of LGL Leukemia

LGL leukemia is a disorder of middle-aged individuals with the median age around 55 years. Both T- and NK-LGL leukemia may manifest as an indolent disorder or an aggressive leukemia. T-LGL leukemia is far more common than NK-LGL leukemia, with more than 85% LGL leukemia manifesting as indolent T-cell disease. Indolent T-LGL leukemia is a chronic disease with median survival of around 14.5 years. On the other hand, aggressive NK-LGL leukemia is one of the most aggressive tumors known, with median survival of a few months following the diagnosis (Loughran 1993; Sokol and Loughran 2006).

About one third of patients with T-LGL leukemia are asymptomatic and are diagnosed coincidentally. Some patients present with B-symptoms such as fever, unexplained weight loss and night sweats. T-LGL leukemia has significant overlap with a variety of hematological and autoimmune diseases. Rheumatoid arthritis (RA) is the most common autoimmune condition seen in the patients with T-LGL leukemia. Recurrent infections due to coexistent neutropenia is a common feature of LGL leukemia. Other hematologic conditions such as hemolytic anemia, pure red cell aplasia, cyclic neutropenia and aplastic anemia also coexist in patients with T-LGL leukemia. While the exact pathogenesis is not understood, it is believed that leukemic T-LGL infiltrate into normal tissues where they mediate direct destruction of normal tissue (Sokol and Loughran 2006; Lamy and Loughran 1998; Zhang et al. 2009).

Treatment of Patients with LGL Leukemia

Asymptomatic LGL leukemia patients with indolent course usually do not require any treatment. Indications for therapy include symptomatic or severe anemia (such as transfusion dependent anemia) or neutropenia (absolute neutrophil count or ANC of <500/μl). The goals of therapy are (i) eradication of clone (ii) correction of

cytopenias and (iii) symptomatic relief. The mainstay of treatment in LGL leukemia is immunosuppressive therapy such as methotrexate (MTX), cyclophosphamide, cyclosporine A (CSA) (Sokol and Loughran 2006; Lamy and Loughran 2003). MTX is one of the most commonly used drugs for the treatment of indolent T-LGL leukemia. Generally, low-dose (~10 mg/m^2), oral treatment is given on weekly basis. The overall response rate (ORR) is about 50%. It may take 3–4 months to observe response. Steroids such as prednisone are sometimes used as adjuvant to hasten the clinical response and symptomatic relief. Due to absence of cross-resistance among MTX, CSA, and cyclophosphamide, trial of other drugs is considered in the event of non-response to the initial treatment. Treatment options such as fludarabine, alemtuzumab (anti-CD52 monoclonal antibody), anti-thymocyte globulin (ATG), pentostatin or splenectomy are considered second-line options in the cases of failure with the first-line treatment (Sokol and Loughran 2006; Osuji et al. 2006).

Pathophysiology of CTL Homeostasis

Activation-Induced Cell Death and Its Dysregulation in LGL Leukemia

In periphery, antigen encounter by an antigen-specific naïve T-cell leads to proliferation of that T-cell. Within days, vigorous proliferation leads to increase in antigen-specific T-cells by about 50,000-fold accompanied by acquisition of effector functions (Thome and Tschopp 2001). Unchecked proliferation and persistent cytotoxicity of CTL is undesirable due to the risk of developing autoimmunity or malignancy. Thus, most of these activated cells are selectively eliminated following antigen clearance (Thome and Tschopp 2001). This process, known as activation-induced cell death (AICD), is paramount for maintenance of T-cell homeostasis and peripheral immune tolerance (Zhang et al. 2004). In the periphery, AICD is achieved by contribution of both granule-mediated pathway and death receptor mediated signaling.

The activation of CTL upregulates the surface expression of both Fas and FasL, ensuring effective elimination of activated CTL via Fas-FasL mediated apoptosis either in autocrine or paracrine fashion (Krammer 2000; Zhang et al. 2004). Fas is a member of tumor necrosis factor receptor (TNFR) family of proteins that plays role in CTL-mediated apoptosis of target cells including other activated CTL (Thome and Tschopp 2001; Matiba et al. 1997). Interaction of FasL with its receptor leads to trimerization of Fas. The cytosolic portion of the trimerized receptor complex binds to an adaptor protein known as Fas-associated death domain (FADD). Collectively, this complex is called death-inducing signaling complex (DISC) (Matiba et al. 1997). Formation of DISC allows for binding and activation of a zymogen known as procaspase-8. Recruitment of procaspase-8 to DISC leads to its cleavage and formation of heterotetramers of two p10 and p18 subunits each (Lavrik et al. 2003). Activated caspase-8 is the key-initiator of death receptor-mediated

apoptosis activating other caspases. The activation of caspase cascade eventually culminates in cell death. DISC formation and the activation of caspase-8 are, in part, negatively regulated by cellular FADD-like IL1-converting enzyme (FLICE)-inhibitory protein (c-FLIP) (Thome and Tschopp 2001).

Dysregulation of Fas-Mediated Apoptosis in Leukemic LGL

Like activated normal CTL, leukemic LGL also express abundant Fas and FasL on their surfaces. However, while normal activated CTL readily undergo Fas-FasL mediated apoptosis, leukemic LGL are resistant to FasL-mediated apoptosis (Lamy et al. 1998).

Gene expression profiling carried out using microarray technique showed a unique gene expression signature in LGL leukemia PBMC. Leukemic LGL show expression pattern in agreement with acquisition of effector functions, but with severe dysregulation in apoptotic machinery. Various genes known to have pro-apoptotic function were downregulated while those with known anti-apoptotic functions were upregulated. Thus, in leukemic LGL the processes of activation and apoptosis that are normally tightly coupled is uncoupled, leading to the inhibition of AICD (Shah et al. 2008).

It is proposed that although leukemic LGL are capable of undergoing Fas-mediated apoptosis, various survival signals keep them from doing so. LGL leukemia patients do not carry any known mutation in Fas or FasL (Liu et al. 2002a). *In vitro* treatment with interleukin-2 (IL2), phytohemagglutinin and IL2, or ceramide sensitize leukemic LGL to Fas-mediated apoptosis (Yang et al. 2008; Epling-Burnette et al. 2001). Inhibitors of various survival signaling pathways restore Fas-sensitivity in leukemic LGL suggesting intact Fas-FasL apoptotic machinery (Shah et al. 2008; Epling-Burnette et al. 2001).

Role of Soluble Fas and FasL in LGL Leukemia

Various isoforms of both Fas and FasL have been found in the sera of LGL leukemia patients. The sera from LGL leukemia patients contained elevated levels of soluble form of Fas receptor (sFas) compared to their healthy counterparts. It is believed that sFas may work as a decoy for FasL, resulting in Fas-resistance phenotype of leukemic LGL (Liu et al. 2002a). In support of this hypothesis, the source of these sFas variants in serum was traced to leukemic LGL. Leukemic LGL were found to express alternative spliced Fas variants not seen in naïve or activated PBMC from healthy controls. When overexpressed, these variants were secreted in supernatant. The supernatant containing these sFas variants blocked Fas-mediated apoptosis of leukemic LGL (Liu et al. 2002a).

Therapeutic Implications

LGL leukemia patients have high amounts of sFasL in serum whereas serum from normal donors does not contain any detectable levels of sFasL. The soluble form of FasL (sFasL) is produced either by alternative splicing or by proteolytic cleavage of membrane bound FasL by matrix metalloproteinase (MMP) family of enzymes (Liu et al. 2002a; Tanaka et al. 1996).

While systemic treatment with Fas agonists such as anti-Fas antibodies or multimeric recombinant FasL seem as a possibility, severe systemic (mainly hepatic) toxicities, preclude their therapeutic use. On the other hand, sFasL is biologically inactive and has activity only when aggregated secondarily by cross linking antibodies. Thus, sFasL can be directed to the target cells by a tumor marker-specific antibody reducing the systemic toxicity while maintaining anti-tumor activity. This approach has shown promise in various leukemia as well as solid tumors (Schrama et al. 2006; Bremer et al. 2008).

MMP inhibitors could theoretically be proposed for the treatment of LGL leukemia, based on the rationale of the inhibition of cleavage of membrane bound FasL resulting in decreased sFasL in the serum. More than fifty MMP inhibitors such as marimastat have been pursued in clinical trials. It would be of interest to see if MMP inhibitors can be used for treatment of LGL leukemia.

Abnormal DISC Formation

DISC formation is the immediate downstream event of Fas-FasL ligation in Fas-mediated apoptosis. Both short and long forms of FLIP (known as c-$FLIP_S$ and c-$FLIP_L$ respectively) contain caspase-homologous regions, enabling them to be recruited to DISC. However, FLIP lack proteolytic capabilities of caspase-8. Thus, recruitment of FLIP to DISC inhibits the execution of Fas-induced apoptosis signals mediated by caspases. FLIP not only inhibit Fas-mediated apoptosis, but is also known to induce NF-κB- and Erk-mediated proliferation in T-cells (Thome and Tschopp 2001). Downregulation of FLIP is seen towards the end of CTL response and correlates with their increased sensitivity to Fas-mediated apoptosis. In contrast, leukemic LGL express higher basal levels of both isoforms of FLIP that may contribute to Fas-resistant phenotype of leukemic LGL (Yang et al. 2008).

Deregulation of Jak-Stat Pathway in LGL Leukemia

Janus kinase-signal transducers and activators of transcription (Jak-Stat) signaling cascade plays role in conferring survival in various tumors. It is also known to be activated following T-cell activation. JAK proteins are kinases that phosphorylate STAT proteins. STAT proteins are latent transcription factors that, upon activation,

transcribe various known anti-apoptotic genes. Jak-Stat pathway plays an essential role following cytokine signaling. Four members of Jak family and seven members of Stat family are known in humans. Stat family members may form homo- or heterodimers resulting in various combinations that may have overlapping but distinct transcription profiles (Murray 2007; Levy and Lee 2002).

Upon activation of cytokine or growth factor receptor, aggregation of receptors leads to transphosphorylation of JAK leading to its activation. Activated JAK can then bind to STAT. STAT proteins contain an N-terminus dimerization domain, a central DNA-binding domain and a C-terminus transactivation domain. Phosphorylation of tyrosine and threonine (believed to be mediated by ERK) of the dimerization domain allows STAT proteins to form homo- or heterodimers. Dimerized STAT proteins then translocate to nucleus where they exert their transcriptional activities by binding to enhancer regions of the target genes including Bcl-xL, myeloid cell leukemia sequence 1 *(*Mcl-1), IAP-family of protein survivin (BIRC5), cell-cycle regulator cyclin D1, c-Myc and vascular endothelial growth factor (VEGF) (Levy and Inghirami 2006). While physiological activation of STATs last for a few minutes to a few hours, constitutively activated STATs are frequently found in a wide variety of human tumors (Steelman et al. 2008). Transformations mediated by oncogenes such as v-*src*, v-*abl*, v-*fps*, v-*fes*, v-*eyk* and $G\alpha_{12}$ have been shown to be mediated by STAT especially by STAT3 (Buettner et al. 2002; Kumar et al. 2005).

Leukemic LGL harbor constitutively activated STAT1 and/or STAT3 but not STAT5. STAT3 and/or STAT1 dimers from LGL leukemia patients' PBMC showed DNA binding activity equivalent to that of *in vitro* activated normal PBMC, suggesting that leukemic LGL are activated *in vivo*. The inhibition of JAK2/3 using small molecular tyrosine kinase inhibitor AG490 induced apoptosis in leukemic LGL as well as restored Fas-sensitivity. Specific inhibition of STAT3 using antisense to *STAT3* induced significant apoptosis as well as restored Fas-sensitivity in leukemic LGL.

The promoter region of human *MCL1* – a BCL2 family member important for maintaining mitochondria integrity – contains a STAT3 binding site. In leukemic LGL, STAT3 binds to this site and induces expression of *MCL1*. The induction of apoptosis through STAT3 inhibition correlates with decreased MCL1 expression indicating a role of anti-apoptotic protein MCL1 in survival of leukemic LGL (Epling-Burnette et al. 2001).

Therapeutic Implications

Recently, there has been a great interest in finding specific methods to inhibit Stat3 signaling. Small molecules inhibitors (JSI124 and platinum (IV) compounds such as CPA-7) or peptide-based inhibitors specific to STAT3 (Iwamaru et al. 2006; Schust et al. 2006; Tan et al. 2006; Turkson et al. 2004) have shown promising results in specifically inhibiting Stat3-mediated signaling.

Stat3 decoy is a double-stranded 15-mer oligonucleotide, corresponding closely to the STAT3 response element (SRE) within the c-fos promoter. Stat3 decoy oligonucleotide binds specifically to activated STAT3 and blocks binding of STAT3 to SREs, acting as a functional antagonist of STAT3 activity in cells. The inhibition of STAT3-mediated transcription is the key to anti-tumor activity. STAT3 decoy has shown to have potent anti-tumor activity *in vitro*, as well as in animal models (Xi et al. 2004). A clinical trial evaluating the efficacy of STAT3 decoy in head and neck cancer is currently ongoing (STAT3 DECOY in Head and Neck Cancer).

Specific STAT3 peptide inhibitors have been developed by fusing the STAT3 SH2 binding domain to a membrane-translocating sequence, which allows delivery of the active peptide in the cells. The STAT3 peptide inhibitor competitively inhibits STAT3 dimerization *in vitro* by direct interaction with STAT3 monomers. Since dimerization of STAT3 is essential for its activity as a transcription factor, these peptides inhibit STAT3 activity by functioning as decoys (Christine et al. 2005).

Small molecular inhibitor OPB-31121, an oral agent, is being evaluated in clinical trials for non-hodgkin's lymphoma, multiple myeloma, and in various advanced solid tumors. OPB-31121 inhibits interleukin-6 dependent phosphorylation of STAT3 thereby exerting its anti-tumor activity (STAT3 Inhibitor for Solid Tumors).

Lestarutinib (CEP 701), an orally available JAK2 inhibitor, is being evaluated in hematopoietic malignancies such as acute myelogenous leukemia and myeloproliferative disorders as well as in prostate cancer, either as a single agent or in combination with other chemotherapeutic agents. Interestingly it is also shown to have activity in autoimmune conditions such as psoriasis (Santos et al. 2010; http://clinicaltrials.gov/ct2/results?term=CEP701).

Trisenox (Arsenic Trioxide, ATO, As2O3) is a highly effective treatment for patients with acute promyelocytic leukemia (APL). It is shown to improve event-free survival and disease-free survival in patients with APL. It is shown to inhibit STAT3 activity in dose-dependent manner by inhibiting the activity of protein tyrosine kinase such as JAK (http://clinicaltrial.gov; Powell et al. 2010; Wetzler et al. 2006).

Given the role of Jak-Stat pathway in survival of leukemic LGL, it will be interesting to see if any of these novel agents would have therapeutic activity in LGL leukemia patients.

Deregulation of Ras-Mek-Erk Signaling in LGL Leukemia

Ras has a well-established role in tumor biology. Mutations of Ras occur in about 30% of all human cancers. Ras family of proteins belongs to guanidine triphosphatase (GTPase). In its active (GTP-bound) form, Ras engages various downstream effector pathways that play essential role in cellular responses such as survival, proliferation and differentiation (Schubbert et al. 2007).

Ras is activated following signal delivered to receptor tyrosine kinases (RTK). Ras signaling can be activated by Src-family kinases (SFK), platelet-derived growth factor (PDGF), or sphingosine-1-phosphate (S1P) through $G\alpha_{12}$. Ras cascade is the major pro-survival regulator following T-cell activation. In T-cells, immunoreceptor tyrosine-based activation motifs (ITAM) of the TCR translate the signal of antigen engagement to Ras (Samelson 2002; Veillette et al. 2002; Pyne and Pyne 2002; Heldin and Westermark 1999; Mor and Philips 2006).

Ras activity is also regulated by post-transcriptional regulations. Prenyl transferases are a class of enzymes that include farnesyl transferases (FT) and geranylgeranyl transferases (GGT). Prenyl-transferases modify Ras activity by adding either one or two hydrophobic moieties on C-terminus of RAS. This modification is essential to anchor Ras on cytosolic leaflet of cellular membranes which is a pre-requisite for activation (Schubbert et al. 2007; Mor and Philips 2006).

Once activated, Ras phosphorylates and activates Raf-1. Raf-1 phosphorylates MAPK-extracellular regulated kinase (ERK) kinase (MEK) resulting in its activation. Activated MEK in turn phosphorylates ERK resulting in its translocation to nucleus. In the nucleus, ERK activates various transcription factors, including Fos and Jun of Ets family, resulting in proliferation, differentiation or survival of cells (Steelman et al. 2008; Schubbert et al. 2007; Mor and Philips 2006). Ras signaling also promotes survival by directly promoting the transcription of FLIP and MCL1 (Budd et al. 2006; Huntington et al. 2007). In Jurkat T-cells MAPK/ERK activity is inversely proportional to Fas-sensitivity and anti-apoptotic activity of MAPK/ERK signaling overrides Fas-mediated apoptotic signals (Holmström et al. 2000).

LGL leukemia patients harbor constitutively active form of Ras (H-Ras-GTP) (Epling-Burnette et al. 2004b). Ras-Mek-Erk signaling was found to be constitutively activated in leukemic LGL. The inhibition of RAS, MEK or ERK induced apoptosis in as well as restored Fas-sensitivity in leukemic LGL (Epling-Burnette et al. 2004b). The inhibition of Ras either using chemical inhibitor FTI2153 or by overexpressing dominant negative form of Ras, induced apoptosis in leukemic LGL by inhibiting ERK activity. Similar results were obtained using MAPK inhibitors (PD98059 or U0216) that induced apoptosis in leukemic LGL and restored Fas-sensitivity in Erk-dependent manner. These results suggest that overactive RAS and MEK lies upstream to ERK in mediating survival signals in leukemic LGL (Epling-Burnette et al. 2004b).

Therapeutic Implications

R1150777 (Zarnestra, Tipifarnib) is a farnesyl transferase inhibitor (FTI) designed to inhibit Ras pathway. Zarnestra is being investigated as a potential treatment in various tumors including leukemia (Armand et al. 2007). Since Ras isoforms such as H-Ras requires farnesylation for malignant transformation activity (Zhu et al. 2005), it was hypothesized that by inhibiting farnesylation of Ras, Zarnestra would inhibit Ras-mediated signaling in LGL leukemia. A clinical trial was conducted on eight

LGL leukemia patients using this drug. While none of the patients achieved clinical response, interesting biological responses were observed in most of these patients.

In a patient with NK-LGL leukemia with coexisting primary pulmonary hypertension (PPH), improvement in the symptoms and signs of pulmonary hypertension following treatment with Zarnestra was observed (Epling-Burnette et al. 2008). Treatment with Zarnestra also resulted in decreased marrow LGL, improved hematopoiesis using *in vitro* cultures, and increased normal marrow hematopoiesis (unpublished observations). It was proposed that activating NKR (or TCR in T-LGL leukemia) acts through adaptor proteins to activate Ras-Mek-Erk and PI3k-Akt signaling. This activation ultimately culminates into Fas-resistance phenotype, granule redistribution, and cytotoxicity. Disruption of Ras signaling using FTI may prevent these downstream effects.

Deregulation of PI3k-Akt Pathway LGL Leukemia

Among downstream effector pathways of Ras cascade, phosphoinositide-3-kinase (PI3k) -v-akt murine thymoma viral oncogene homolog (Akt) mediated signaling has a well established role in metabolism, survival and proliferation – and hence in tumor formation. This deregulation is commonly caused by mutation or amplification of PI3K-α, or deletion or mutation of a negative regulator of PI3k-Akt signaling, phosphatase and tensin homolog (PTEN) (Vivanco and Sawyers 2002).

In T-cells, PI3k-Akt signaling plays pivotal role in TCR-mediated activation and proliferation. PI3K is activated upon membrane relocation. This relocation is mediated by the interaction of PI3K with RAS or SFK, or through the direct interaction of PI3K with cytokine receptors at the SH2-domain binding site (Steelman et al. 2008; Vivanco and Sawyers 2002; Schade et al. 2006). The most studied component downstream of PI3K is a serine/threonine kinase – AKT (or protein kinase B, PKB). The activation of AKT accounts for many of the biological functions of PI3K. To promote proliferation, AKT acts through upregulating cyclin D1, and mammalian target of rapamycin (mTOR) pathway, while downregulating forkhead box class O (FOXO) transcription factors and cyclin-dependent kinase inhibitors (CKI) such as $p21^{WAF}$ and $p27^{KIP1}$.

One of the main targets downstream to Akt in pro-survival signaling is NF-κB signaling. Further, AKT phosphorylates Bcl-2 antagonist of cell death (BAD), preventing its interaction with anti-apoptotic factor Bcl-x_L. This leaves Bcl-x_L to exert its anti-apoptotic functions. AKT phosphorylates and inhibits caspase-9, thus inhibiting apoptosis. Another anti-apoptotic phosphorylation target of AKT is MDM2 – a negative regulator of tumor suppressor p53. Phosphorylated MDM2 binds to p53, expediting its degradation and interfering with tumor suppression effects of the latter (Wymann and Schneiter 2008; Mayo and Donner 2001).

In LGL leukemia PBMC, SFK maintain PI3K in its constitutively active form as assessed by phosphorylated state of AKT and glycogen synthase kinase-3 (GSK3). Following activation, PI3k-Akt signaling can enhance the survival of normal T-cells

through the inhibition of Fas clustering and DISC (Jones et al. 2002), a phenotype consistent with leukemic LGL. Further, the inhibition of SFK or PI3K induced apoptosis in leukemic LGL. The inhibition of SFK or PIK3 was accompanied by the inhibition of ERK1/2 activity, placing Akt upstream to Erk in LGL survival signaling. In leukemic LGL, SFK-mediated activation of PI3k-Akt pathway results in constitutive ERK activity. The inhibition of this pathway at any level – namely the inhibition of SFK, AKT or ERK –interferes with the survival of leukemic LGL resulting in induction of apoptosis (Epling-Burnette et al. 2004b; Schade et al. 2006).

Therapeutic Implications

Due to their role in promoting proliferation and survival as well as potent anti-angiogenic effects, PI3k and Akt are well established candidates for anti-tumor therapy. Currently, many pan-PI3K inhibitors are being studied in pre-clinical and clinical trials. The strategies to use PI3k-Akt signaling inhibitors are either to use them as single agents or in combination with conventional chemotherapy or radiotherapy. Among many isoforms, PI3Kα is the most commonly involved isoform in cancer. Hence, as a strategy to minimize side effects, efforts are underway to develop a PI3Kα specific inhibitor. Currently, inhibitor of PI3K such as PI-103, NVP-BEZ235, SF1126, PX-866, and ZSTK474 (all pan-PI3K isoform inhibitors), have shown promise as anti-tumor agents as well in the treatment for autoimmune diseases. Given this dual effect, it would be of interest to see if these novel agents possess therapeutic potential in treatment of LGL leukemia (Wymann and Schneiter 2008; Kong and Yamori 2008).

Deregulation of NF-κB Signaling in LGL Leukemia

NF-κB plays an essential role in hematopoiesis, inflammation, as well as survival and proliferation of adaptive immune system cells. Thus, NF-κB is now recognized as a critical player in almost all the aspects of immune responses (Sen and Baltimore 1986; Hayden et al. 2006). In inactivated state, NF-κB is in cytoplasm as a complex with the inhibitor of NF-κB (IκB). This complex keeps NF-κB from both entering to nucleus and binding to DNA, thus depriving its transcriptional functions. This inhibition is void once IκB is phosphorylated by (IκB)-kinase complex (IKK). Phsophorylation of IκB, leads to its ubiquitination and proteasomal degradation. IKK is a known AKT substrate. In summary, phosphorylation of IKK by AKT leads to the activation of the IKK, which in turn leads to phosphorylation and degradation of IκB, leading to NF-κB activation (Wymann and Schneiter 2008).

The activation of NF-κB is one of the most characterized pathways in antigen-receptor signaling in T-cells. The activation of NF-κB downstream of TCR ligation facilitates proliferation and acquisition of effector functions. NF-κB orchestrates T-cell activation by providing a milieu to proliferate (such as inducing IL2 production)

and acquire effector functions (such as promoting transcription of RANTES, Fas and FasL) (Hoffmann and Baltimore 2006).

An important NF-κB function is to protect T-cells against AICD (Rivera-Walsh et al. 2000). This function is executed through promoting the expression of pro-survival Bcl-2 family member and inhibitor of apoptosis (IAPs) proteins (Hoffmann and Baltimore 2006). Deficiency or inhibition of NF-κB activity results in failure of activation, either due to lack of proliferation or premature onset of apoptosis suggesting critical role of NF-κB signaling in mounting effective T-cell response (Hayden et al. 2006; Kontgen et al. 1995; Jeremias et al. 1998; Wan and DeGregori 2003).

Due to its pivotal role in CD8+ T-cell activation and survival along with an established role in various aspects of tumorigenesis as well as inflammation, NF-κB is an interesting candidate to study in LGL leukemia. Gene expression data of leukemic LGL showed that c-Rel was overexpressed in leukemic LGL (Shah et al. 2008). Further, leukemic LGL showed constitutively active NF-κB. The inhibition of NF-κB using a specific inhibitor BAY 11-7082 resulted in apoptosis of leukemic LGL. The inhibition of Akt led to the inhibition of NF-κB activity; whereas, the inhibition of NF-κB activity did not affect Akt phosphorylation. This suggests that NF-κB acts downstream of PI3k-Akt pathway in leukemic LGL. We also found that NF-κB maintains expression of Mcl-1 independent of STAT3 activity (Zhang et al. 2008).

Therapeutic Implications

NF-κB is identified as a major player in cell growth, proliferation, immunity and transformation, making it an attractive target treatment of various diseases. However, due to its widespread role in cell biology, the inhibition of NF-κB signaling is also prone to significant toxicities. Bardoxolone methyl (CDDO-Me, RTA402), a synthetic triterpenoid, is an oral inhibitor that is known to inhibit two key signaling pathways implicated in survival of leukemic LGL: NF-κB- and Jak/Stat- pathways. CDDO-Me inhibits tumor necrosis factor-α (TNFα)-induced phosphorylation of IKKβ (a component of IKK complex) thus inhibiting translocation of NF-κB to the nucleus thus inhibiting NF-κB signaling. CDDO-Me also blocks interleukin-6 (IL6)-induced and constitutive STAT3. CDDO-Me binds directly to STAT3inhibiting the formation of STAT3 dimers (Ahmad et al. 2006, 2008). CDDO-Me is being tested in clinical trials for the treatment of both lymphoid malignancies and solid tumors (RTA-402).

Deregulation of the Sphingolipid Rheostat in LGL Leukemia

Sphingolipids are biologically active lysophospholipids that act either directly or as second messengers to regulate diverse biological functions such as survival, proliferation, and migration. Ceramide (N-acyl sphingosine), a pro-apoptotic sphingolipid, can be synthesized either *de novo* (by condensation of serine and palmytoyl-CoA) or by catabolic pathway (by breaking down sphingomyelin). Ceramide is synthesized

in cells following wide variety of stress or death signals, including Fas-FasL interaction. Ceramide can be deacylated into sphingosine by ceramidases such as acid ceramidase (ASAH1) or phosphorylated to ceramide-1-phosphate (C1P) by ceramide kinase. Sphingosine, then, can be phosphorylated by one of the two sphingosine kinases (SPHK1 or SPHK2) into sphingosine-1-phosphate (S1P). Though structurally related to ceramide, S1P is a pro-survival molecule. Given that pro-apoptotic (such as ceramide and sphingosine) and anti-apoptotic (such as S1P and C1P) sphingolipids exist in a rapidly exchanging equilibrium, it has been proposed that the relative amounts rather than absolute quantities of these molecules, determine the cell fate – this equilibrium is known as the sphingolipid rheostat (Wymann and Schneiter 2008; Rosen and Goetzl 2005; Spiegel and Milstien 2002).

Role of sphingolipids, especially that of S1P, in oncogenesis, metastasis and angiogenesis is well established (Visentin et al. 2006). S1P acts in autocrine or paracrine manner, either intracellularly or through one of the five highly specific S1P receptors (S1PR) on the cell surface – $S1P_1$ through $S1P_5$ (Spiegel and Milstien 2002). S1PRs belong to a class of receptors known as G-protein coupled receptors (GPCR). GPCR are coupled with G-proteins that act as relay junction for transmitting extracellular signals to various signaling pathways. One such G-protein relevant to S1P biology is $G\alpha_{12}$. The roles of three components of sphingolipid rheostat are well studied – sphingosine kinase, acid ceramidase, and G-protein $G\alpha_{12}$. It was shown that sphingolipid rheostat is dysregulated in leukemic LGL. This altered sphingolipid rheostat is believed to confer survival on leukemic LGL. It was shown that ASAH1 and SPHK were upregulated in leukemic LGL. It was also shown that Galpha12-mediated signaling was upregulated in leukemic LGL. Further, leukemic LGL expressed different pattern of S1PR compared to their healthy counterparts. Finally, disruption of the sphingolipid rheostat induced apoptosis in leukemic LGL but not in LGL derived from healthy controls.

SPHK is overexpressed in variety of tumors and is considered an oncogene (Spiegel et al. 1998; Milstien and Spiegel 2006). The activation of various survival signaling pathways implicated in tumorigenesis such as platelet-derived growth factor (PDGF) or vascular endothelial growth factor (VEGF) result in the activation of SPHK. SPHK in turn activates many of these pathways constituting a positive feedback loop. Persistent elevation of phosphatidylinositol (3,4,5) trisphosphate results in the activation of SPHK linking PI3k-Akt cascade to sphingolipid signaling. Similarly, ERK activation leads to the activation of SPHK linking Ras-Mek-Erk signaling to sphingolipid rheostat. Activated SPHK feeds into several proliferative and pro-survival pathways by activating ERK, PI3K and NF-κB. SPHK promotes cell survival by increasing cellular concentration of S1P while reducing concentrations of ceramide and sphingosine. This puts SPHK in unique position where various survival signaling pathways converge, rendering it an interesting candidate for anti-tumor therapy.

Acid ceramidase functions upstream to SPHK in sphingolipid metabolism. Upregulation of ceramidase is a survival mechanism used by variety of human tumors to combat ceramide production that normally follows various apoptosis-inducing insults (Spiegel et al. 1998; Kolesnick 2002; Park and Schuchman 2006). N-oleoylethanolamine (NOE), a chemical inhibitor of acid ceramidase, selectively induces apoptosis in various types of tumor cells (Morales et al. 2006).

$G\alpha_{12}$ is coupled with S1PRs $S1P_1$ and $S1P_5$. Constitutive active form of $G\alpha_{12}$ is sufficient to induce transformation that is at least partially mediated by activating STAT3. The components believed to play a role in $G\alpha_{12}$-mediated transformation include JAK3, PDGFα, and PI3K (Kumar et al. 2005). All these components are known to be deregulated in LGL leukemia suggesting involvement of $G\alpha_{12}$ in survival of leukemic LGL (Shah et al. 2008; Epling-Burnette et al. 2001; Schade et al. 2006; Zhang et al. 2008).

S1P receptor $S1P_1$ is the most predominant S1PR in human naïve CD8+ T-cells, while other receptors are expressed at very low levels. Following activation, $S1P_1$ and $S1P_5$ are further downregulated in normal CD8+ cells (Shah et al. 2008). In contrast to normal CD8+ T-cells, $S1P_5$ is the predominant S1PR on leukemic LGL. $S1P_1$ is constitutively downregulated in LGL leukemia PBMC suggesting that leukemic LGL are activated *in vivo* (Shah et al. 2008; Brinkmann 2007).

In T-cells, S1P plays a role by protecting cells against ceramide and Fas-FasL mediated apoptosis (Wymann and Schneiter 2008; Cuvillier et al. 1998; Goetzl et al. 1999). S1P protects T-cells against Fas-mediated apoptosis in various T-cell lines as well as in human PBMC (Shah et al. 2008; Cuvillier et al. 1998; Goetzl et al. 1999). Microarray analysis showed that sphingolipid- and $G\alpha_{12}$-mediated signaling was enriched in leukemic LGL. *ASAH1* was identified as a core enriched component in leukemic LGL. In agreement with Fas-sensitive phenotype of activated normal PBMC, ASAH1 is downregulated to undetectable levels following activation in normal PBMC. In contrast, leukemic LGL express abundant ASAH1 presumably facilitating the breakdown of ceramide resulting in the Fas-resistant phenotype observed in leukemic LGL (Lamy et al. 1998; Shah et al. 2008).

As discussed above, sphingolipid rheostat is dysregulated in leukemic LGL. Hence, it was hypothesized that the disruption of the sphingolipid rheostat in a way that tilts the balance in the favor of pro-apoptotic molecules (such as ceramide) and away from anti-apoptotic molecules (such as S1P) should lead to induction of apoptosis in leukemic LGL. Indeed, the inhibition of ASAH1 using its chemical inhibitor NOE induced significant apoptosis in leukemic LGL (Shah et al. 2008). Similarly, the inhibition of SPHK using chemical inhibitors (SKI-I and SKI-II) led to induction of apoptosis in leukemic LGL (Zhang et al. 2008). Further, the inhibition of S1P-mediated signaling using FTY720 led to induction of apoptosis in leukemic LGL but not in LGL isolated from healthy controls. FTY720 treatment also restored Fas-sensitivity in leukemic LGL suggesting a role of S1P-mediated signaling in protection against AICD (Shah et al. 2008). Collectively, these results suggest the role of sphingolipid rheostat in the pathogenesis of LGL leukemia.

Therapeutic Opportunities

FTY720 or Fingolimod is a novel compound in clinical trials as immunomodulator in post-renal transplant patients, in patients with allergic conditions such as asthma, and in those with autoimmune conditions such as multiple sclerosis (MS). FTY720 is structurally similar to S1P and following phosphorylation, can bind to four out of

five S1PR (except $S1P_2$) acting as a functional antagonist (Brinkmann 2007). Given that FTY720 selectively induces apoptosis of leukemic LGL and that LGL leukemia patients often have coexisting autoimmune diseases, it is possible that FTY720 may have a therapeutic role in these patients.

Sphingosine kinase inhibitors, SKI-I and SKI-II, are known to have anti-tumor activity and have proven their anti-tumor activity in mouse models (French et al. 2006). Development of newer, more selective inhibitors targeting SPHK offer exciting therapeutic opportunities (Paugh et al. 2008).

Neutralizing antibody to S1P has been proposed as a candidate for blocking survival and angiogenic signaling in various tumors. It works as a 'sponge' to absorb extracellular S1P – leading to abolition of S1P-mediated protection against apoptosis (Milstien and Spiegel 2006). However, there are no reports of neutralizing antibody to S1P as a potential in therapeutic in leukemia. It remains to be seen if it might have activity in LGL leukemia.

Ceramide is a well established mediator of apoptosis *in vitro* and *in vivo*. However, the use of ceramide as a therapeutic agent is stymied by its insolubility. Liposome-based drug delivery system has been proposed as a mean to overcome this therapeutic barrier. It was recently shown that treatment with ceramide containing nanoliposomes resulted in complete remission in rat model of aggressive NK-LGL leukemia. Polyethylene glycol coated liposomes, called pegylated liposomes, were shown to facilitate entry of ceramide into the cells. Nanoliposomes that are formulated at 80 ± 15 nm in size and contain 30 mol% cell-permeable ceramide were shown to be much less toxic than the delivery of 'free' ceramide both *in vitro* and *in vivo*.

Following delivery in nanoliposomal form, ceramide accumulated in mitochondria of leukemic NK cells resulting in caspase-dependent apoptosis of leukemic NK-cells both *in vitro* and *in vivo*. This was accompanied by the inhibition of ERK activity and downregulation of survivin expression.

To test the therapeutic benefit of nanolipsomal ceramide *in vivo*, Fischer F344 rat LGL leukemia model were treated either with ghost or C6-ceramide nanoliposomes. It was shown that treatment with ceramide containing nanoliposomes conferred significantly prolonged survival in addition to normalization of LGL counts in the blood, bone marrow, lymph nodes and lungs. Given the safety profile in various animal models, ceramide containing nanoliposomes may be an important therapeutic modality in patients with LGL leukemia. (Liu et al., *in press*) With the availability of technique to coat nanoliposomes with antibodies, it is tempting to predict the advent of targeted nanoliposomes such as those targeting only CD3+ cells. Such a treatment modality would be expected to achieve greater efficacy at lower dose with fewer side effects.

The Role of Interleukin-15 Signaling in LGL Leukemia

Recently, network theory approach was used to identify the most important regulators in the survival of leukemic LGL. First, a survival signaling network was constructed by integrating signaling pathways involved in normal CTL activation

and the known deregulations of survival signaling in leukemic T-LGL. Then, the network was translated into a predictive discrete dynamic Boolean model. By simulating node deregulations corresponding to known signaling deregulations, it was concluded that the constitutive presence of interleukin-15 (IL15), the presence of PDGF, and the initial T-cell activation signal are sufficient to reproduce all known deregulations in leukemic T-LGL. IL15 and PDGF were shown to play a crucial roles in the survival of leukemic LGL, experimentally validating predictions made using network modeling approach (Zhang et al. 2008).

The generation of memory lymphocytes depends on antigenic stimulation. The survival of these memory lymphocytes requires cytokines such as IL2 and IL15. Even though IL2 and IL15 share two subunits of their heterotrimeric receptors, their role in CTL homeostasis is contrasting – while IL2 facilitates AICD, IL-15 promotes proliferation and long-term survival of memory phenotype CD8+ T-cells in an antigen-independent fashion. Il15 also promotes survival of NK cells *in vivo* (Waldmann 2006; Liu et al. 2002b). Given its role in survival of both memory T-cells and NK-cells it was not unexpected that IL15 has a pivotal role in the pathogenesis of both forms of LGL leukemia. The inhibition of IL15 signaling (by blocking IL15 or the unique subunit of IL15 receptor complex, IL15Rα) induces apoptosis of leukemic LGL and increases the levels of BH3-interacting domain death agonist (Bid) in leukemic LGL, suggesting role that Bid plays in abnormal survival of leukemic LGL (Hodge et al. 2009).

Bid is a BH3-only member of Bcl-2 family of proteins. Bid is a proteolytic substrate for activated caspases -3 and -8 in death receptor signaling pathway as well as for granzyme B. Following cleavage, truncated Bid (tBid) translocates to the outer mitochondrial membrane to participate in mitochondrial permeabilization, thus acting as a sentinel for protease-mediated death signaling. This places Bid in a unique position connecting death-receptor signaling pathway to mitochondrial pathway of cell death (Billen et al. 2008).

In normal NK cells IL15 upregulates E3 ligase HDM2. HDM2 directly interacts with Bid and significantly reduces Bid accumulation by enhancing proteasomal degradation of the latter. Bid levels are low in both leukemic T- and NK-LGL compared to their normal counterparts. It was further shown that forced overexpression of Bid using transduction approach leads to enhanced apoptosis of T-LGL leukemia cells (Hodge et al. 2009).

Therapeutic Implications

Bortezomib (PS341, Velcade) is a proteasomal inhibitor that is used in the treatment of various cancers such as multiple myeloma and mantle cell lymphoma. Bortezomib is an inhibitor of 26 S proteasomal complex, a large multi-subunit complex that degrades ubiquinated proteins in eukaryotic nucleus and cytosol. Bortezomib induces apoptosis in a wide variety of cancer cell lines with relatively few toxic effects on their normal counterparts. By inhibiting proteasomal pathway, Bortezomib interferes with numerous signaling pathways (Mitchell 2003).

The treatment of leukemic T- or NK-LGL with Brotezomib led to induction of apoptosis in these cells. Bortezomib also induced the expression of Bid. It was proposed that Bortezomib induces the expression of Bid which is followed by cleavage of Bid into tBid. tBid, then, proceeds with its pro-apoptotic activity. Bortezomib also induced Fas or TRAIL-independent apoptosis in leukemic LGL, leading to a hypothesis that Bid induced apoptosis may be explained by its role in DNA damage and repair (Hodge et al. 2009).

Deregulation of PDGF Signaling in LGL Leukemia

PDGF is a major growth factor and mitogen for various cell types. A molecule of PDGF is a dimer made up of structurally similar A, B, C, and D polypeptide chains, which combine to four homodimeric (PDGF-AA, PDGF-BB, PDGF-CC and PDGF-DD) and one heterodimeric PDGF-AB proteins. PDGF is synthesized by many different cell types, and its expression is broad. The PDGF isoforms exert their cellular effects by binding to two structurally related receptor subunits, denoted the α-receptor and the β-receptor. Three PDGF receptor (PDGFR) complexes PDGF-αα, PDGF-ββ and PDGF-αβ mediate overlapping but not identical signaling. PDGF-α and PDGF-β belong to type III–receptor tyrosine kinase (RTK) families and are characterized by five immunoglobulin-like domains in the extracellular region, a transmembrane domain, an ATP-binding site, and a hydrophilic kinase insert domain in the cytosol. The activation of PDGFR complex can activate many major signal transduction pathways, including the Jak-Stat, PI3k-Akt, and Ras-Mek-Erk signaling pathways. The activation of PDGFR thus results to downstream effects such as stimulation of cell growth, DNA replication, angiogenesis, and wound healing.

The *sis* oncogene of simian sarcoma virus (SSV) was found to encode the B-chain of PDGF more than two decades ago. SSV transformation involves autocrine stimulation by a PDGF-like molecule. Since then, dysregulation of PDGF signaling has been implicated in various malignancies including leukemia, gastrointestinal stromal tumors (GIST), glioblastoma multiforme (GBM), dermatofibrosarcoma protruberans, and in myeloproliferative diseases such as chronic myelomonocytic leukemia (CMML). In addition, overproduction of PDGF is implicated in autocrine and paracrine growth stimulation of various tumors (Heldin and Westermark 1999; Alvarez et al. 2006).

As described above, the presence of enhanced IL15 and PDGF signaling is sufficient to reproduce all known deregulations in T-LGL leukemia (Zhang et al. 2008). In this sense, PDGF can be considered one of the master switches in orchestrating survival signaling in T-LGL leukemia. Platelet-poor plasma from patients from both forms of LGL leukemia had about threefold higher levels of circulating PDGF-BB compared to their normal counterparts. The source of PDGF-BB was traced back to leukemic LGL. Interestingly, PDGF signaling operates in autocrine nature as leukemic LGL overexpress PDGFR-β transcripts compared to their

normal counterparts. Sera from patient with LGL leukemia results in enhanced autophosphorylation of PDGF-RTK in leukemic LGL. Enhanced PDGF signaling results in constitutive activation of Sfk-PI3k-Akt and Mek-Erk signaling pathways in leukemic LGL. The inhibition of SFK activity (using chemical inhibitor PP2) or PI3K activity (using chemical inhibitor LY294002) inhibited PDGF-BB-induced activation of AKT and ERK in both the forms of LGL leukemia, resulting in the induction of apoptosis. Finally, the inhibition of PDGF-β-RTK (using chemical inhibitor AG1296) induces apoptosis in leukemic LGL (Zhang et al. 2008; Yang et al. 2010).

The direct effect of PDGF signaling on proliferation of leukemic LGL was demonstrated when the addition of PDGF-BB led to sixfold increase in cell numbers compared to sera from normal counterparts. This PDGF-BB dependent proliferation of leukemic LGL could be blocked by inhibition of PDGF-β-RTK, SFK, or PI3K. Neutralizing antibody to PDGF-BB inhibited cell proliferation in the leukemic LGL in PI3k-Akt dependent manner, placing PDGF signaling upstream to PI3k-Akt in LGL leukemia survival signaling (Yang et al. 2010).

Therapeutic Implications

Imatinib (STI-571, Gleevec) is a RTK-inhibitor that inhibits tyrosine kinases such as PDGFR, ABL and KIT at therapeutic concentrations *in vivo*. Imatinib has been successfully tried in patients with myeloproliferative disease involving dysregulation of PDGFR signaling (Apperley et al. 2002). While the anti-neoplastic role of imatinib is well established, recent publications suggest its usefulness in chronic inflammation and autoimmune conditions such as scleroderma and arthritis (Paniagua et al. 2006; Chung et al. 2009). While the exact mechanism of action of imatinib in autoimmune conditions is not known, it may be hypothesized that the inhibition of PDGF signaling as occurs in leukemic LGL may play a role. Given the safety profile of imatinib and the fact that PDGF-β is implicated in the survival of leukemic LGL, it would be of interest to see if imatinib can be used as a potential therapeutic strategy for patients with both T- and NK-LGL leukemia.

Concluding Remarks

LGL leukemia is a rare lymphoproliferative disorder of cytotoxic cells. The mainstay of treatment for LGL leukemia as discussed above is immunosupression. While drugs such as MTX and CSA are effective in many patients, the current modalities of treatment are not curative. The problems with such conventional therapies include the long duration of treatment with many patients receiving the treatment indefinitely, side effects including the possibility of second malignancy, and frequent recurrence. Further, in most studies complete hematological response (CHR) was achieved

far less common than partial response (PR) indicating residual disease. Given these problems, it is imperative that more targeted therapy for LGL leukemia be developed.

Given the cytotoxic potential of CTL and NK cells, it is hardly any surprise that LGL leukemia is associated with various autoimmune disorders. Due to this close association with variety of autoimmune diseases, it is proposed that therapy aimed at leukemic LGL may also be of use in patients with autoimmune diseases. Since its first description, many survival mechanisms in leukemic LGL are unraveled. With development of more specific small molecular inhibitors, oligonucleotides, peptides, and monoclonal antibodies, it will be of great interest to see evolution of the therapeutic armamentarium for patients with LGL leukemia.

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