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ROLE OF GSH METABOLISM IN MEDIATING STROMAL-LEUKEMIA INTERACTION AND PROMOTING CELL SURVIVAL AND DRUG RESISTANCE IN CHRONIC LYMPHOCYTIC LEUKEMIA

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ROLE OF GSH METABOLISM IN MEDIATING STROMAL-LEUKEMIA INTERACTION AND PROMOTING CELL SURVIVAL AND DRUG RESISTANCE IN CHRONIC LYMPHOCYTIC LEUKEMIA

A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

Wan Zhang, M.S.

Houston, Texas

May, 2011

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Publication No.

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Supervisory Professor: Peng Huang, M.D., Ph. D.

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the western countries. The interaction between CLL cells and the bone marrow stromal environment is thought to play a major role in promoting the leukemia cell survival and drug resistance. My dissertation works proved a novel biochemical mechanism by which the bone marrow stromal cells exert a profound influence on the redox status of primary CLL cells and enhance their ability to sustain oxidative stress and drug treatment. Fresh leukemia cells isolated from the peripheral blood of CLL patients exhibited two major redox alterations when they were cultured alone: a significant decrease in cellular glutathione (GSH) and an increase in basal ROS levels. However, when cultured in the presence of bone marrow stromal cells, CLL cells restored their redox balance with an increased synthesis of GSH, a decrease in spontaneous apoptosis, and an improved cell survival. Further study showed that CLL cells were under intrinsic ROS stress and highly dependent on GSH for survival, and that the bone marrow stromal cells promoted GSH synthesis in CLL cells through a novel biochemical mechanism. Cysteine is a limiting substrate for GSH synthesis and is chemically unstable. Cells normally obtain cysteine by uptaking the more stable and abundant precursor cystine from the tissue environment and convert it to cysteine intracellularly. I showed that CLL cells had limited ability to take up extracellular cystine for GSH synthesis due to their low expression of the transporter

Xc-, but had normal ability to uptake cysteine. In the co-culture system, the bone marrow stromal cells effectively took up cystine and reduced it to cysteine for secretion into the tissue microenvironment to be taken up by CLL cells for GSH synthesis. The elevated GSH in CLL cells in the presence of bone marrow stromal cells significantly protected the leukemia cells from stress-induced apoptosis, and rendered them resistant to standard therapeutic agents such as fludarabine and oxaliplatin. Importantly, disabling of this protective mechanism by depletion of cellular GSH using a pharmacological approach potently sensitized CLL cells to drug treatment, and effectively enhanced the cytotoxic action of fludarabine and oxaliplatin against CLL in the presence of stromal cells. This study reveals a key biochemical mechanism of leukemia-stromal cells interaction, and identifies a new therapeutic strategy to overcome drug resistance *in vivo*.

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LIST OF ABBREVIATIONS

CLL…….……Chronic lymphocytic leukemia

GSH……...……..Glutathione

F-ara-A……...…9-β-D-arabinofuranosyl-2-fluoro-adenine

PEITC……..β-phenylethyl isothiocyanate

NAC……...…….N-acetylcysteine

H_2O_2……...…….Hydrogen peroxide

PI……Propidium iodide

DTNB……...……..5,5'-dithiobis-(2-nitrobenzoic acid)

GCLC……γ-glutamylcysteine ligase catalytic subunit

GCLM……...γ-glutamylcysteine ligase modulatory subunit

(S)-4-CPG…….(S)-4-carboxyphenylglycine

SAS……...Sulfasalazine

HMW……..……..High molecular weight

LMW…….…….Low molecular weight

2-ME……...…2-Mercaptolethanol

MOLDI-TOF MS…….....

……Matrix-assisted laser desorption/ionization- time of flight mass spectrometer

ESI-MS/MS…… ……..Electrospray ionization tandem mass spectrometry

1H-NMR……....Proton nuclear magnetic resonance

ROS…….... Reactive oxygen species

siRNA…….…… Small interference RNA

CM……..…….……Conditioned medium

MeAIB……..……α-methylamino-isobutyric acid

DHB……...Dihydroxy- benzoic acid

α-CHC……...α-cyano-4-hydroxycinnamic acid

INTRODUCTION

1. Role of microenvironment in drug resistance of chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL), the most common adult leukemia in the western countries, was last reviewed in the *New England Journal of Medicine* in 2005 [1], this review updated new information about CLL based on the understanding of the biology and derivation of CLL cells during the past 10 years. Progress in CLL study further underlines the role of leukemia microenvironment to support CLL cells survival and drug resistance. The current views of CLL point out that, firstly, rather than inherent apoptotic defect in the entire mass of leukemia cells, CLL cell accumulation occurs more likely because of survival signals delivered from microenvironment to a subset of leukemia cells. Secondly, the studies of BCR-triggering capacities [2, 3], surface membrane phenotypes [4], telomere lengths [5], gene-expression profiles [6], and *in vivo* measurement of cell division rates [7], indicate that, CLL is a disease of accumulating B lymphocytes with higher proliferation than previously thought. While inducing anti-apoptotic signal, the mediators in the leukemia microenvironment also render the CLL cells apoptosis-prone following correlating signaling pathway activation [8]. The machinery of apoptosis is functional in CLL cells [9]. To resist apoptosis, CLL cells get "help" from leukemia microenvironment to balance the pro- and anti- apoptotic signals in favor of cell survival. This hypothesis is well proven by the phenomenon that anti-apoptotic CLL cells *in vivo* quickly go spontaneous apoptosis *in vitro*, which can be largely prevented by co-culturing with stromal cells [3]. Thirdly, the discovery of new prognostic markers in CLL nicely indicates the promoting role of accessory signals from microenvironment. CLL is a clinically heterogeneous disease that currently categorized by the mutation status of V

genes, expression of ZAP70 and CD38 [10-12]. Unmutated V genes reflect high antigen binding possibility, while ZAP70 and CD38, as the intracellular and surface protein respectively, both are involved in signal transduction and activation of CLL cells. CLL cells that carry these markers always have unfavorable disease progress and short survival. Although the exact functions of these markers are not clear yet, it indicates that relatively active CLL cells, which dynamically interact with the leukemia microenvironment, are more likely to survive and associated with aggressive disease. Fourthly, the residual marrow nodules are the major sites of treatment failure in clinic [13]. CLL originates in the peripheral lymphoid organs, bone marrow is the major target of CLL cells. It has been recognized that the interactions of CLL cells with components of bone marrow and lymph node allow the subpopulation of cells to survive initial drug treatment, the expansions of these cells further acquire drug resistance and finally cause relapse [14].

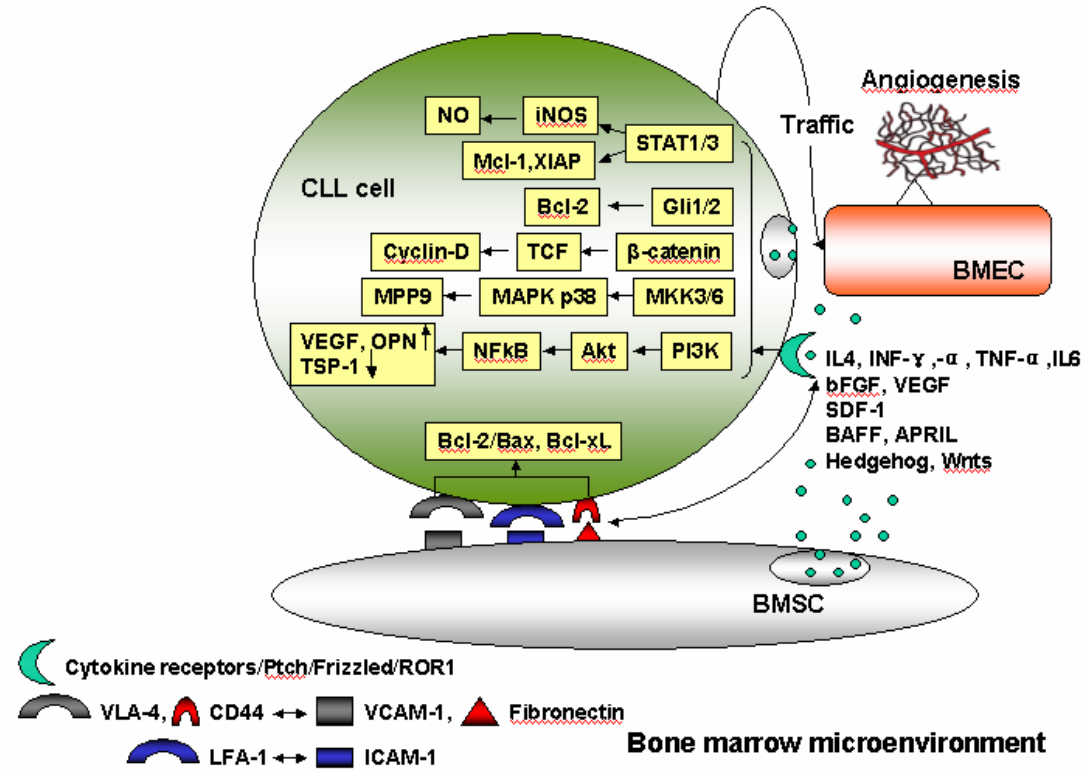
In the recent years new therapeutic strategies such as fludarabine-based regimens have significantly improved the treatment outcomes for patients with CLL, the most common adult leukemia in the United States and Europe [13]. However, failure to eliminate the residual leukemia cells that are resistant to drug treatment and the eventual reemergence of the leukemia cell population continue to be a major clinical challenge, and CLL remain as an incurable disease [13, 15]. Although many anticancer drugs are effective in killing CLL cells in vitro, the leukemia cells are much more resistant to drug treatment in vivo. Growing evidence suggests that the bone marrow stroma may provide a tissue environment that promotes the survival of CLL cells and render drug resistance [1, 14]. Patients with CLL often present with bone marrow infiltration [16, 17], and the expansion

of the residual CLL cells after drug treatment eventually leads to disease relapse and treatment failure. Thus, understanding the mechanisms by which the tissue microenvironment promotes leukemia cell survival and drug resistance is critical for developing new therapeutic strategies to specifically abrogate such protective mechanisms and effectively eliminate the malignant cells in vivo.

2. Stromal-leukemia interaction

The interactions of CLL cells with bone marrow stromal cells through cell-cell contact and soluble factors could activate molecular pathways that promote survival and drug resistance of CLL cells (**Fig. 1**).

Figure 1. Stromal-CLL interactions activate molecular pathways in CLL cells and promote survival and drug resistance of CLL cells.



(1) Cell adhesion mediated survival and drug resistance in CLL cells.

The adhesion molecule expression pattern of CLL cells is quite complicated. CLL cells constitutively express β1 and β2 integrin, together with variable α3, α4, α5 integrins. CLL cells have variable expression of lymphocyte function-associated antigen 1 (LFA-1), very late antigen-4 (VLA-4), inter-cellular adhesion molecule 1 (ICAM-1/CD54), ICAM-2 (CD102), ICAM-3 (CD50), and L-selectin (CD62L) [18, 19]. CD44 is also detected in certain aggressive population [20, 21]. Besides mediating migration of CLL cells to their niche in bone marrow and secondary lymphoid tissues [18, 22], some of these adhesion molecules also protect CLL cells for survival and confer drug resistance through binding to their receptors on stromal cells. For example, β1 and β2 integrins on CLL cells acting simultaneously mediate binding to ICAM-1 (CD54) and VCAM-1 (CD106) on bone marrow stromal cells, and prevent apoptosis of CLL cells correlating with preventing loss of Bcl-2 protein expression; while normal B cells cannot be protected by stromal cells due to lack of this adhesion pattern [23, 24]. An independent group also observed the role of β2 integrin in the prevention of apoptosis induction in CLL cells [25]. Moreover, the interaction of VLA-4 (α4β1 integrin) on CLL cells with fibronectin produced by stromal cells prevents apoptosis and induces resistance to fludarabine, correlating with elevated Bcl-2/Bax ratio and Bcl-x_L level [26, 27]. Another adhesion molecule that might be involved in CLL survival is CD44, high CD44 expression was found on an aggressive subtype of CLL patients [28]. In multiple myeloma cells, CD44 mediates binding to bone marrow stromal cells through fibronectin and VCAM-1, CD44/ fibronectin binding upregulates p27, activates NFkB and confers cell adhesion-mediated drug resistance of multiple myeloma cells [29]. Cell adhesion-mediated drug resistance is widely observed in multiple

myeloma cells [30-32], it remains to be assessed whether similar molecule pathways are also activated in CLL cells.

(2) Soluble factor mediated survival and drug resistance in CLL cells.

It is well known that bone marrow stromal cells secrete various cytokines including stromal cell-derived factor-1 (SDF-1), IL6, IL4, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin-like growth factor 1 (IGF1), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-α, –γ (INF-α, –γ), tumor necrosis factors α (TNF α), TNF family members CD40L, B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL). Protection effect of these cytokines on CLL cells have been shown by experiments of specifically adding exogenous cytokines and relevant antibodies to CLL cells. For example, exogenous IL4, INF-α and –γ inhibit spontaneous apoptosis and promote CLL cells survival *in vitro*, which are significantly reduced by their antibodies [33]. The mechanism studies have identified some survival pathways of these cytokines. IL4, INF-α, –γ and bFGF prevent apoptosis of CLL cells using a Bcl-2 dependent pathway [34-39]. In addition, IL4 and INF–γ also upregulate the expression of inducible nitric oxide synthase (iNOS) in CLL cells and cause endogenous release of NO, which contributes to anti-apoptosis through S-nitrosylation thus suppression of caspase 3 [40, 41]. STAT1 and 6 might play important role in the cytokine-induced iNOS expression [40]. VEGF is another important stromal cell-derived factor that not only protects CLL cells survival but also induces angiogenesis. On one hand, by binding to VEGFR, VEGF activates downstream STAT 1 and 3 in CLL cells, and thus upregulates the expressions of Mcl-1 and XIAP that enhance apoptotic resistance [42]. On the other hand, VEGF as

well as bFGF play a central role in angiogenesis. Recently, the role of angiogenesis in CLL cells has been recognized to influence disease progression through producing even more powerful cytokine interactions among CLL cells, bone marrow stromal cells, and endothelial cells [43, 44].

Besides cytokines, other stromally secreted proteins also have been identified to mediate stromal protection in CLL cells. After stromally induced hedgehog signaling pathway being reported in lymphoma and multiple myeloma cells, this pathway has also been identified in CLL cells co-cultured with stromal cells. Hedgehog protein activates its receptor PTC on CLL cells, which then releases Smo. Smo activation leads to transcriptional activity of Gli1 and 2 that finally upregulate the expression of Bcl-2 [45]. Wnt is another stromally secreted protein that contributes to anti-apoptosis of CLL through binding to Frizzled receptor and activating Wnt/β-catenin signaling [44]. Especially, Wnt5a can also bind to orphan receptor tyrosine kinase (ROR1), the signature surface receptor tyrosine kinase of CLL cells, induces activation of NF-κB [45], the newly discovered pair of Wnt5a/ROR1 might enhance the capacity of CLL cells to receive survival signals from microenvironment and confer drug resistance.

In fact, the leukemia microenvironment is complicated. Now people more and more agree that the cell adhesion molecules and soluble factors usually talk with each other, enhance each other, and collaboratively mediate survival and drug resistance in CLL cells. Moreover, the migration of CLL cells to the bone marrow milieu especially needs the crosstalk of integrin and cytokines [18, 22].

(3) Other activated survival pathways in CLL cells by unknown mediators from stromal cells.

There are some other signaling pathways are activated in CLL cells by interacting with bone marrow stromal cells, while the mediators from the stromal cells are still unknown. For example, the signaling pathway of MKK3/6/ MAPK p38/ MMP9 is activated in CLL cells when co-cultured with stromal cells, which induces anti-apoptosis and angiogenesis [46]. Another upregulated pathway in co-cultured CLL cells is PI3K/NF-kB, which prevents the downregulation of Bcl-x_L expression, induces expression of XIAP and $FLIP_L$ and finally inhibits caspase3 activation and apoptosis [47]. Using DNA microarray technology, another group confirmed this pathway by identifying a number of genes in PI3K/Akt/NFkB pathway in CLL cells induced by BMSCs. They further proved that this pathway mediated upregulation of pro-angiogenic molecules vascular endothelial growth factor (VEGF) and osteopontin (OPN) and downregulation of anti-angiogenic molecules thrombospondin-1 (TSP-1) [48].

Despite the progress of studying the molecular interaction of stromal cells and CLL cells, none of these soluble factors or adhesion moleculars alone or in combination could mimic the bone marrow microenvironment, indicating the complicity of the stromal environment. A better understanding of the biology of stromal-CLL interaction is urgently needed to guide the design new therapeutic strategies.

3. Oxidative stress of chronic lymphocytic leukemia and glutathione

Studies of CLL cells including basal superoxide levels, mitochondrial DNA mutation, oxidative DNA damage and antioxidant enzyme activities have shown that, compared to normal lymphocytes, CLL cells exhibit increased production of reactive oxygen species (ROS) and are under oxidative stress [49-52]. This has been further proven by the fact that CLL cells are quite sensitive to ROS-mediated anticancer agents [49, 52].

Moreover, B-CLL cells are more susceptible to H_2O_2 than normal lymphocytes [53]. All these indicate that CLL cells are highly dependent on anti-oxidant system to maintain redox balance.

Glutathione (GSH), the chief nonprotein intracellular sulfhydryl, is the major antioxidant that maintains a redox balance in the cellular compartments. Besides removing endogenous free radical, increased GSH levels largely affect the efficacy and interactions of a variety of antineoplastic interventions. The mechanisms that contribute the GSH-mediated drug resistance include: (1) Defense against oxidative stress produced by ROS generating drugs. (2) Drug inactivation and alterations in drug transport; (3) Increased repair and tolerance of DNA damage; (4) Apoptosis inhibition.

(1) ROS scavenger: Several anticancer agents currently used for cancer treatment have been shown to cause increased cellular ROS generation [54]. In cancer cells under oxidative stress, overproduction of ROS by those drugs not only lead to irreversible cell injury, but also exhaust the capacity of antioxidant defense [55, 56]. The electrophilic properties of GSH enable it to react with H_2O_2 nonenzymatically or by the action of glutathione peroxidase(GPX), yields GSSG [57]. Cancer cells that have high levels of GSH are recognized to fulfill the protective function, and survive the exogenous ROS.

(2) Drug inactivation and alterations in drug transport: GSH is widely recognized to protect cells via inactivation and elimination of cytotoxic agents. GSH-drugs adducts may form spontaneously or the process may be catalyzed by Glutathione S-transferases (GSTs) in a greater rate [58]. Glutathione S-conjugates are often more hydrophilic and less toxic, they can be transported outside the cells through GS-X pump, an ABC transporter

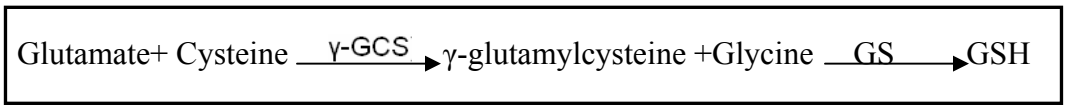
family member coded by MDR gene [59, 60]. Thus, both GST and GS-X pump have important roles in GSH-mediated drug resistance [60, 61].

(3) Increased repair and tolerance of DNA damage: DNA is a well established target of antitumor drugs, cells with enhanced ability of DNA repair can survive and gain alkylator chemoresistance [62]. There's accumulating evidence indicates that GSH has an additionally potential to facilitate DNA repair [63], either through diverting folates to *de novo* purine and pyrimidine synthesis [64], or through formation of less toxic lesions that are easily repaired [65, 66]. In general, facilitating DNA repair can have an impact in GSH-mediated resistance.

(4) Apoptosis inhibition: GSH can significantly affect cell survival. For example, cytochrome c induces apoptosis only in its oxidized form, while elevated GSH keeps it in an inactive state (reduced form) and thus prevents apoptosis [67]. GSH can also inhibit apoptosis through mechanisms not directly through modulating ROS levels [68]. For instance, glutathionylation of pro-caspase-3 renders it resistance to protolytic cleavage and thus prevents caspase activation [69]. In CLL cells, glutathionylation of the anti-apoptotic protein MCL1 protects it from being cleaved by caspase-3 and thus promotes cell survival [52]. A recent study indicated that GSH may promote lymphoid cell survival through maintaining intracellular ionic homeostasis [70]. With its nucleophilic nature, GSH may conjugate with electrophilic drugs, promote their export from the cells, and thus decrease the efficacy of many anticancer drugs [71]. GSH can also reduce the activity of oxaliplatin by decreasing the ROS stress induced by the drug [72]. These compelling evidences suggest the important role of GSH in cell survival and drug resistance.

4. Glutathione metabolism

GSH concentration in cells reflects the dynamic balance between the rate of GSH synthesis, consumption (through multiple functions described above), and efflux [73, 74] (**Fig 2**). GSH synthesis is a two-step enzymatic process involving the ligation of glutamate with cysteine to form γ-glutamylcysteine catalyzed by γ-glutamylcysteine ligase (GCL), and the addition of glycine to the C-terminal of γ-glutamylcysteine catalyzed by GSH synthetase (GSHS) to form glutathione tripeptide [73]:



The synthesis of γ-glutamylcysteine is a rate-limiting step catalyzed by the rate limiting enzyme γ-glutamylcysteine ligase (GCL) [73]. GCL composed of catalytic subunit GCLC (heavy-73KD) and modulatory subunit GCLM (light-30KD). Increased expression of the GCLC is correlated with elevated GSH levels and drug resistance in tumor cells [74, 75].

Another important rate-limiting factor in GSH synthesis is the availability of the substrate cysteine. Unlike glutamate, cysteine concentration in cells approximates the Km value of GCLC [76] (**Table 1**), indicating that the speed of GSH synthesis cannot reach maximal and highly depend on cellular concentration of cysteine. Cysteine is a conditionally essential amino acid which can be synthesized from methionine only in certain tissues such as liver via the transsulfuration pathway [77], but many tissues including lymphoid cells have little capacity to synthesize cysteine due to a defect in transsulfuration [78]. Thus, their main source of cysteine is the uptake of extracellular cysteine or cystine through specific transporters [79-81]. Cysteine is transported by the ubiquitously expressed ASC transporter (Na^+-dependent) as well as the Na^+-independent

transporters [82], while cystine is mainly transported by Xc-, which is limited expressed, and can be rapidly reduced to cysteine for GSH synthesis once inside the cells [83-87]. Paradoxically, in plasma, cystine presents at 100-200 μM concentrations, while cysteine only exists at a much lower level in the range of 10-20 μM due to oxidation to cystine easily [88] (**Fig. 3**).The limited cysteine in plasma seems not enough for GSH synthesis in those cancer cells that are under oxidative stress but lack of expression of cystine transporter Xc-. Therefore, activated cysteine uptake, increased cysteine concentration in the microenvironment, and regained of cystine transport activity will influence GSH levels as well as drug resistance [89-93].

In addition, cells can also re-use cysteine from GSH through the γ-glutamyl cycle catalyzed by γ-glutamyl transpeptidase (GGT) [94]. GGT is an enzyme on the outer surface of plasma membrane, it catalyzes the transfer of the the γ -glutamyl group of GSH to acceptors. Cysteine is the most active acceptors. Cysteinylglycine, formed in the transpeptidation reaction, is split by dipeptidases to cysteine and glycine, and then cysteine can be re-used. The γ -glutamatyl-amino acids formed by transpeptidation are substrates of γ -glutamyl cyclotransferase, which converts them into 5-oxoproline and the corresponding amino acids. Finally, conversion of 5-oxoproline to glutamate is catalyzed by 5-oxoprolinase. In this case, the glutamate can be reused.

GSH is readily oxidized to GSSG non-enzymatically or catalytically by glutathione peroxidase (GPX), GSSG efflux from cells contributes to the loss of cellular GSH [95]. Oxidative stress may cause changes in the GSH redox state and increase the rate of GSSG release from cells [96]. Moreover, exportation of the GSH-electrophiles conjugated

products (catalyzed by GSH S-transferase---GST) results in an irreversible loss of GSH [95].

Figure 2. GSH biochemistry. aa, amino acids; x, molecules that bind GSH forming conjugates; ROS, reactive oxygen species; (1) GGT; (2) γ -glutamyl amino acid (γ -Glu-aa) transporter; (3) dipeptidases; (4) Cyst(e)ine transporters; (5) γ -Glu-cyclotransferase; (6) 5-oxoprolinase; (7) GCS; (8) GSHS; (9) GPx; (10) GR; (11) transhydrogenases; (12) GSTs; (13) GSSG efflux; (14) conjugated product efflux

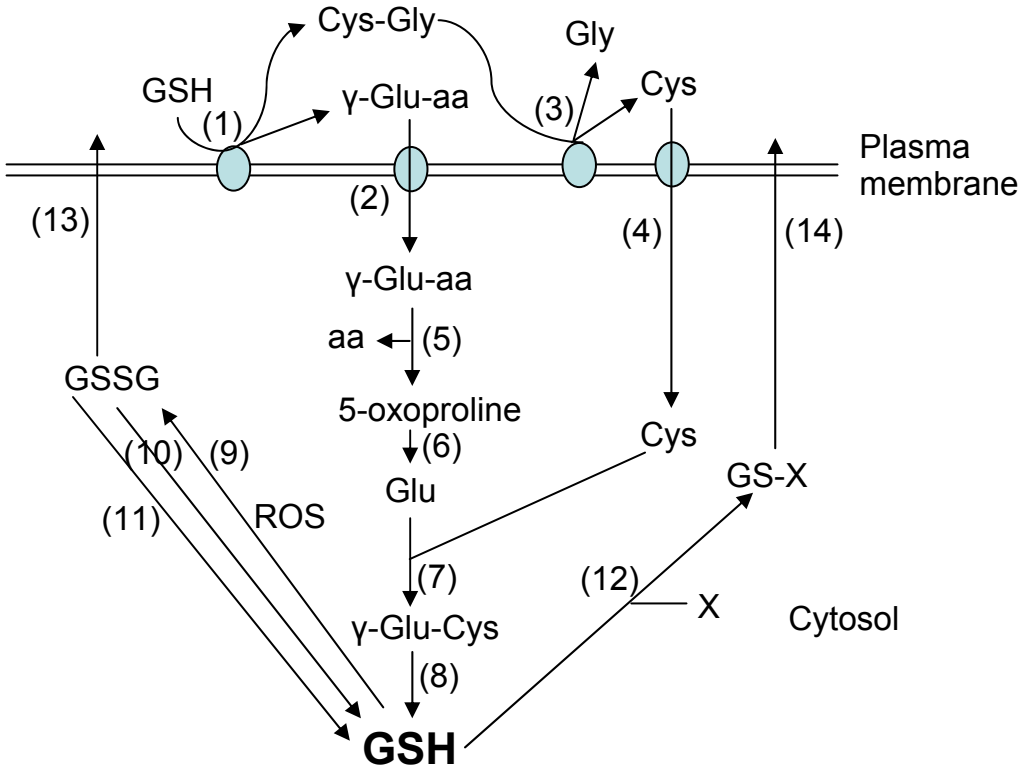
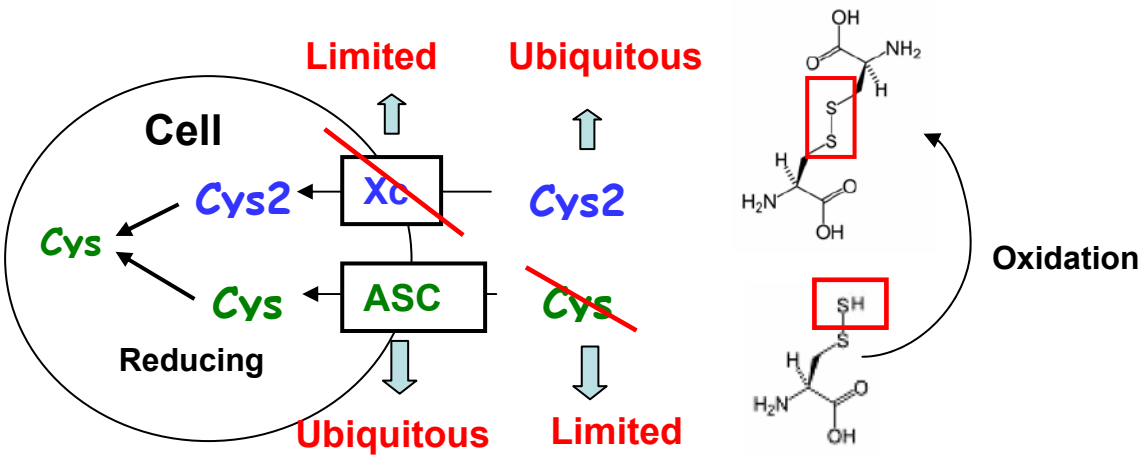

Cys-Gly
Gly
GSH
γ-Glu-aa
(1)
(3)
Cys
Plasma
membrane
(13)
(2)
(4)
(14)
γ-Glu-aa
aa
(5)
GSSG
5-oxoproline
(6)
Cys
(10)
(9)
Glu
GS-X
(11)
ROS
(7)
(12)
X
Cytosol
γ-Glu-Cys
(8)
GSH

Table 1. Km value of γ-glutamylcysteine ligase catalytic subunit (GCLC) and cellular concentration of glutamate and cysteine (*adapted from Richman, P. G., Meister, A., J Biol Chem1975 1975. 250(4): p. 1422-6.*)

	γ-glutamylcysteine ligase catalytic subunit Km	Cellular concentration
Glutamate	1.8mM	18mM
Cysteine	100-300uM	≤300uM

Figure 3. Cystine and cysteine transportation in cells.



5. Glutathione and chronic lymphocytic leukemia

CLL cells are under intrinsic oxidative stress as compared to normal lymphocytes [49-51], and are quite sensitive to ROS-mediated anticancer agents [49, 52]. Therefore, CLL cells are highly dependent on the most abundant antioxidant GSH to maintain cellular redox balance. Moreover, GSH plays important role in blocking apoptosis of CLL cells. In CLL cells, Glutathionylation of the anti-apoptotic protein MCL1 protects it from being cleaved by caspase-3 and thus promotes cell survival [52]. Furthermore, GSH, as discussed above, causes drug resistance of CLL cells, which is the major reason of relapse and treatment failure.

In CLL cells, while GSH is so important, CLL cells seem not be able to maintain GSH by itself. An interesting report showed that, when CLL cells were cultured *in vitro*, there was a significantly rapid decrease in cellular GSH concomitant with spontaneous apoptosis of CLL cells [97]. The rapid GSH depletion was not observed with the T cells from CLL patients or with either B or T cells from normal subjects indicating that this phenomenon is unique to CLL cells. It would be important to study why CLL cells fail to maintain GSH *in vitro* and go spontaneous apoptosis quickly, and how the *in vivo* leukemia microenvironment maintain the GSH level of CLL cells thus support the cells for survival.

STATEMENT OF OBJECTIVES

CLL is the most common adult leukemia in the western countries and is currently incurable [15]. Growing evidence suggests that the bone marrow stroma may provide a tissue environment that promotes the survival of CLL cells and render drug resistance [1, 14]. Several molecules have been identified to be involved in the interaction of bone marrow stromal cells and CLL cells, such as adhesion molecules β1 and β2 integrins on the CLL cell surface [23], and the stromal-produced CD40L [98], IL-4 [34], INF-α [35], INF–γ [36], bFGF [37], SDF-1 [99], BAFF [100], APRIL [100], hedgehog-related molecules [45]. However, bone marrow microenvironment induced drug resistance and relapse is still a major clinical problem [13]. New therapeutic strategies are urgently needed, and there will most likely result from a better understanding of the biology of stromal-leukemia interaction.

Recent technological developments have allowed global analyses of biochemical alterations in cancer, and enabled the discovery of the potential roles of low-molecular-weight metabolites in cancer development [101, 102]. Studies suggest that stromal cells may interact with cancer cells at the biochemical levels [103-107]. In CLL, it remains largely unknown how the stromal cells may affect leukemia cells metabolically and promote their survival and drug resistance.

Among those small molecules, glutathione (GSH) plays a unique role in CLL. CLL cells are intrinsically under high oxidative stress compared to the normal lymphocytes [49-51], and are highly sensitive to agents that cause further ROS stress [49, 52]. The elevated ROS in CLL cells renders them more dependent on antioxidants GSH to maintain redox balance. However, CLL cells, but not normal lymphocytes, fail to maintain GSH level *in vitro* concomitant with high level of spontaneous apoptosis in culture. In contrast, CLL

cells are known to have a prolonged survival time *in vivo* and are less prone to apoptosis compared to normal lymphocytes when they reside within the tissue environment *in vivo* [108].

These observations, together with the important role of GSH in maintaining redox balance and promoting cell survival, led me to hypothesize that the stromal tissue environment affect the GSH metabolism in CLL cells and thus influence their survival and apoptotic response to drug treatment. Based on the hypothesis, experiments have been performed to achieve the following three specific aims:

- Aim 1: Test the important role of GSH in microenvironment-mediated protection of CLL cells.

- Aim2: Examine the mechanisms by which stromal cells regulate intracellular GSH levels in CLL cells and protect CLL cells from spontaneous and drug-induced cell death.

- Aim 3: Determine the feasibility and significance of targeting the mechanisms of microenvironment mediated GSH upregulation and protection in CLL cells as an effective way to kill CLL cells and circumvent drug resistance.

The major goal of this study is to test the hypothesis using a co-culture system in which the possible biochemical communications between primary leukemia cells isolated

from CLL patients and bone marrow stromal cells was investigated, and the subsequent changes in GSH, cell viability, and drug sensitivity were evaluated. This study reveals a novel biochemical mechanism that mediates the interaction between the bone marrow stromal cells and leukemia cells through enhancing glutathione synthesis to promote CLL cell survival and drug resistance. Importantly, I have identified a pharmacological approach that can effectively abolish this protective mechanism and sensitize CLL cells to standard drug treatment in the presence of stromal cells. The new mechanistic insights gained from this study provide a biochemical basis for developing new therapeutic strategy to overcome CLL drug resistance *in vivo*.

EXPERIMENTAL MODEL AND METHODS

1. Experimental model

Unlike other types of leukemia, there are no available valid cell lines for studying CLL. Fortunately, through collaboration with hematologists in MD Anderson cancer hospital, I have access to obtain blood from clinical patients with CLL. To test my hypothesis, I used an *in vitro* co-culture model that primary CLL cells isolated from samples of patient peripheral blood, were cocultured with a human bone marrow stromal cell line HS5, a HPV16 E6/E7 immortalized cell line, or StromaNKtert, a hTERT immortalized cell line, or with a mice immortalized cell line KUSA-H. The ratio of stromal cells and CLL cells is 1:20. This model is used to mimic the interaction of CLL cells with bone marrow microenvironment and study the mechanism of drug resistance.

2. Reagents and antibodies

9-β-D-arabinofuranosyl-2-fluoro-adenine (F-ara-A), Oxaliplatin, and β-phenylethyl isothiocyanate (PEITC) for toxicity assay; N-acetylcysteine (NAC), Cysteine, Cystine, 2-mercaptolethanol (2-ME) (all from Sigma-Aldrich) for cellular redox modulation; Serine, α-methylamino-isobutyric acid (MeAIB), Sulfasalazine (all from Sigma-Aldrich), and (S)-4-carboxyphenylglycine (Ellisville, MO) for transporter inhibition; Glutathione assay kit (Cayman Chemical Co.) for GSH detection; siRNA (Invitrogen) for xCT knockdown; Human cytokine array kit (R&D System) for cytokine detection in culture medium; CM-H_2DCF-DA (Invitrogen) for hydrogen peroxide detection; CM-FDA (Invitrogen) for thiol detection; Annexin V-FITC (BD Biosciences), propidium iodide (PI) (Sigma-Aldrich) for apoptosis assay; 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Sigma-Aldrich) for medium thiol detection; [14C]-cystine (PerkinElmer) for uptake assay; Cystine-free

PRMI 1640 (Mediatech) and dialyzed FBS (Thermo Scientific HyClone) for cystine starvation assay. The following antibodies were used for immunoblotting analyses using standard Western blotting procedures: GCLC (Santa Cruz Biotechnology), SLC7A11 (Novus Biologicals), Actin (Sigma-Aldrich).

3. Cell lines and primary CLL cells

The human bone marrow stromal cell line HS5 immortalized by E6/E7 [109], was obtained from the American Type Culture Collection. Human bone marrow stromal cell line StromaNKtert immortalized by hTERT [110] and the murine bone marrow stromal cell line KUSA-H1[111] were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum as described previously [112]. Primary CLL cells were obtained from the peripheral blood of patients diagnosed as B-CLL according to National Cancer Institute Criteria [113]. Proper informed consents under a research protocol approved by the Institutional Review Board of M. D. Anderson Cancer Center were obtained from all patients in accordance with the Declaration of Helsinki before blood sample collection. In all experiments, CLL cells were isolated from blood samples by density gradient centrifugation [114] and incubated in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin (100 U/mL) + streptomycin (100ug/mL) overnight before experiments.

4. Cell viability assays

To determine the effect of bone marrow stromal cells and conditioned medium on CLL cells against drug- and stress-induced apoptosis, HS5 ($5x10^4$ cells/mL), StromaNKtert ($4x10^4$ cells/mL), and KUSA-H1 ($1x10^4$ cells/mL) were seeded in 24-well plates and allowed to adhere and grow overnight before addition of CLL cells. The

stromal conditioned medium was prepared from HS5 cells culture (in RPMI 1640+10% FBS for 5 days), clarified by centrifugation, and used immediately. CLL cells were isolated from blood samples and incubated overnight, and then transferred to 24-well plates with or without pre-seeded stromal cell layer or stromal conditioned medium. For co-culture in a transwell system (Corning, NY), CLL cells were seeded in the top chambers, which were inserted into the bottom wells with pre-seeded stromal cells. After co-culture for 1 day, CLL cells were treated with various compounds (F-ara-A, oxaliplatin, PEITC, H_2O_2) under conditions indicated in the figure legends. To block cystine uptake by the stromal cells, (S)-4-carboxyphenylglycine (Ellisville, MO) or Sulfasalazine (Sigma) was added at the beginning of the stromal cell seeding. Cell viability was determined by flow cytometry after double-staining with Annexin V (BD Biosciences, San Jose, CA) and PI as described [115]. All assays were repeated at least three times using primary CLL cells from different patient samples.

5. Flow cytometric analysis of cellular ROS and thiol contents

After cultured under the indicated conditions, CLL cells ($1x10^6$ cells/ml) were transferred to 24-well plate and incubated with 1 μM CM-H2DCF-DA (Invitrogen, Carlsbad, CA) in regular culture medium for 60 min at 37 °C in the dark. Stained cells were rinsed twice with PBS and analyzed immediately by flow cytometry using a FACSCalibur equipped with CellQuest Pro software. To determine cellular thiol contents, CLL cells (1x106 cells/ml) were collected and washed in PBS, and stained with 0.5 μM CMFDA (Invitrogen, Carlsbad, CA) in PBS for 15 minutes at room temperature in the dark, followed by flow cytometry analysis. ROS levels in viable cells were

determined by FSC/SSC gating as described previously (Campas et al., 2003; Pepper et al., 2001).

6. Separation stromal conditioned medium into high molecular weight and low molecular weight components

HS5 stromal cells were cultured in RPMI1640 medium with 10% FBS until 80% confluence. The cells were washed twice with serum-free RPMI 1640, and then cultured in serum-free medium for 3 days. The conditioned medium was harvested, cleared by centrifugation, and then loaded to the reservoir chamber of the Amicon Ultra-15 centrifugal filter unit with molecular weight cut-off of 3-kDa (Millipore corporation, Billerica, MA). The tube was centrifuged at 4000x g at 4°C for 15min. The concentrated liquid remaining in the upper reservoir (HMW fraction) was collected and reconstituted with serum-free medium to same volume before centrifugation. The components that passed the filter were collected as LMW fraction. Both fractions were used immediately after preparation.

7. Western blot analysis

Cell lysates were prepared and equal amounts of protein were electrophoresed on SDS-PAGE gels using standard conditions. The proteins were transferred to nitrocellulose membranes, which were probed with the following antibodies: GCLC (Santa Cruz Biotechnology, Santa Cruz, CA); xCT (Novus Biologicals, Littleton, CO); actin (Sigma-Aldrich, St Louis, MO). Protein bands were visualized by chemiluminescent detection.

8. Assessment of requirements for exogenous cystine

Bone marrow stromal cells (HS5, StromaNKtert, or KUSA-H1) was plated and allowed to adhere for overnight as indicated in the figure legends. CLL cells were isolated from blood samples and incubated overnight. The culture medium of both cells was removed, followed by a rinse with warm PBS. CLL cells were then resuspended in cystine-free RPMI 1640 (Mediatech, Manassas, VA) supplemented with 10% dialyzed FBS (Thermo Scientific HyClone, Logan, Utah) and added to the culture dish containing pre-washed HS5 stromal cells. Various concentrations of cystine (5-200 μM), 2-mercaptoethanol (20 μM), and their combination were added to the culture as indicated in the figure legends. Cellular GSH and cell viability were measured as described above.

9. Assessment of cystine and cysteine uptake

To measure cystine uptake, CLL and HS5 cells alone or in co-culture were incubated in fresh cystine-free RPMI 1640 supplemented with dialyzed 10% FBS. [14C]-cystine (PerkinElmer, Waltham, MA) was at (0.2 μCi/ml) and incubated for 4-6 h as indicated. The cells were washed twice with cold PBS. Cell pellets were resuspended in 200 μL PBS, lysed with 3 mL scintillation fluid, and radioactivity was measured by a Beckman liquid scintillation counter. To measure cysteine uptake, [14C]-Cysteine was first generated by reduction of [14C]-cystine using 5 mM 2-mercaptoethanol (37°C, 15 min) in a test tube with minimum volume, and then add to the cell culture. [14C]-Cysteine uptake was measured in the same way as described above. All experiments were performed in triplicates.

10. Analysis of glutathione (GSH) in cell extracts and in culture medium

GSH was measured using an assay kit from Cayman Chemical (Ann Arbor, MI), based on the enzymatic recycling catalyzed by glutathione reductase and the reaction of

GSH with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to produce a yellow colored product, which can be quantified by a spectrometer. After CLL cells were cultured under various experimental conditions, they were collected, sonicated, de-proteinated, and assayed for GSH according to the procedures recommended by the manufacture. The culture medium was cleared by centrifugation, deproteinated, and subjected to the same GSH assay. GSH concentrations were calculated using the standard curve generated in parallel experiments. To avoid the influence of other thiol in medium, both the end-point method and kinetic methods were used for analysis. For the endpoint method, GSH concentration was calculated from the reading of a 25-min reaction. For the kinetic method, the slopes of the absorbance changes (at 405 nm) were obtained for a range of standard GSH concentrations to generate a standard curve (Slops vs concentrations). The GSH concentrations in the tested samples were calculated from their respective slopes in reference to the standard curve. Data were obtained from triplicate measurements. To determine the minimum detection limit of the assay to test GSH in cell culture medium, various concentrations of standard GSH was added to RPMI medium containing 10% FBS and processed for detection of GSH using the kinetic method described above.

11. Determinations of thiol concentration in the culture medium

HS5 (5x 10^4 cells/mL), StromaNKtert (4x 10^4 cells/mL), and KUSA-H1 (1x 10^5 cells/mL) cells were plated in T75 flasks and allowed to adhere overnight. CLL cells were isolated from blood samples and also incubated in regular RPMI medium (1x 10^6 cells/mL) overnight. The old medium of each culture was replaced with fresh medium. At the indicated time intervals, 300 μL medium was removed, clarified by centrifugation, and mixed with 100 μL of 15% sulfosalicylic acid. The precipitates were removed by

centrifugation (7000 rpm, 15min), and the supernatant was neutralized with 200 μL 0.6 N NaOH and mixed rapidly with 600 μL buffer containing 0.2 M potassium phosphate and 10 mM EDTA, pH 8.0. Thiol contents were measured by reaction with freshly prepared DTNB (Sigma-Aldrich, St Louis, MO) at a concentration of 0.4 mM. Each sample was assayed in triplicate. Absorbance at 412 nm was measured after 5-min incubation in dark, and the increase in absorbance was determined. The acid-soluble thiol concentration was calculated using cysteine as a reference standard. Medium alone without cells was incubated in parallel at the same time as a control. Data were obtained from triplicate measurements.

12. Determination of oxygen effect on CLL cell viability

Primary leukemia cells were isolated from the blood samples of CLL patients, incubated overnight in RPMI medium, and transferred to 24-well plates with or without pre-seeded stromal cells. The samples were incubated in ambient oxygen (21%) or hypoxia (5%, 2%) for 24 h, then the cells were treated with F-ara-A (20 μM) or oxaliplatin (20 μM) for 48 h. Cell viability was determined by flow cytometry after double-staining with annexin-V/PI. Hypoxia culture conditions were created by incubating cells in a sealed modular incubator chamber (Billups-Rothenberg, Del Mar, CA) flushed with a gas mixture containing 5% or 2% oxygen and 5% carbon dioxide, balanced with nitrogen.

13. Cytokine array

Secreted cytokines were detected using the human cytokine array panel A from R&D systems (Minneapolis, MN). CLL cells and HS5 stromal cells were cultured alone or co-cultured for 3 days. Cell culture supernatants were collected and particulates were

removed by centrifugation and assayed immediately according to the procedures recommended by the manufacture. Medium alone was examined as control.

14. MALDI-TOF mass spectrometry analysis

MALDI-TOF/TOF-MS/MS experiments were performed on a MALDI TOF-TOF Mass Spectrometer (Applied Biosystems 4700, Foster City, CA). HS5 stromal cell conditioned medium were diluted 1:100 in α-cyano-4-hydroxyl cinnamic acid (a-CHC) (10 mg/mL in 50:50 acetonitrile:water; 0.1% trifluoracetic acid final concentration) or dihydroxybenzoic acid (DHB) by mixing 50% volume of 10 mg/mL dihydroxybenzoic acid (DHB) in acetonitrile and 50% volume of 0.1% trifluoroacetic acid (TFA) in water, and spotted on the MALDI target, and allowed to dry before analysis. MS experiments were acquired using the reflectron settings in the positive mode. MS spectra were summed from 1000 to 10 000 laser shots.

15. ESI-MS/MS analysis

Pure cysteine prepared in water and stromal cell conditioned medium were injected to mass spectrometry. Mass spectrometry was carried out in positive ion mode on a linear ion trap mass spectrometer, using a nanoelectrospray source for direct infusion of samples by static nanospray with isolation width as m/z 1.0, and acquisition time as 0-8 min. Electrospray voltage was 1 kV. Static nanoelectrospray needles were from Proxeon Biosciences (Denmark).

16. NMR analysis of low-molecular-weight metabolites in cells and culture medium

To measure possible changes in cystine, cysteine, and other small metabolites in the cell culture medium, the stromal conditioned medium and fresh medium were collected and sent to Chenomx Inc (Edmonton, Canada) for NMR analysis. All samples were

filtered using the Nanosep 3K Omega microcentrifuge filter tubes (Pall Corporation, NY, USA) with a 3-kDa molecular weight cut-off to remove macromolecules. The filter units were washed 3 times with distilled water before use. Samples were adjusted to 630 μL to ensure adequate volume for NMR acquisition, and mixed with an internal standard solution (Chenomx Lot# 01-28-09-01, contains 4.4729 mM DSS, 0.2 %w/v NaN3, 10 mM DFTMP in D2O) that allows metabolite quantification. Mixed solution was then transferred to a 5 mm NMR tube (New Era Enterprises Inc., NJ, USA) for data acquisition. All metabolite concentrations obtained were adjusted by using appropriate factors to account for the above dilutions. Spectra were acquired on an 800 MHz Varian INOVA spectrometer equipped with a Z-gradient HCN 5mm cold-probe (Varian Inc., CA, USA). Spectra were processed and CNX files were generated using the Processor module in Chenomx NMR Suite 6.0 software (Chenomx, Edmonton, Alberta, Canada). Spectra were zero filled to 64k points and Fourier transformed. Spectral phasing was performed on the spectra along with baseline correction (Chang et al., 2007). Metabolites were identified and quantified with targeted profiling approach using the Profiler and Library Manager modules in the same software which contained 297 metabolites and 54 typical plasma based metabolites (Weljie et al., 2006). Minimum detection limit was approximately 2 μM.

17. Analysis of Cysteine by LC-MS/MS on the Triple-Quadrupole Mass Spectrometer

The chromatography was performed on a Zorbax SB-C18 Rapid Resolution HD column, 3.0 x 100 mm, 1.8 micron particle size from Agilent. Buffers used were as follows: A was 0.5% formic acid, 0.3% heptafluorobutyric acid in HPLC-grade water

(Burdick and Jackson); B was 0.5% formic acid, 0.3% heptafluorobutyric acid in HPLC-grade (Burdick and Jackson) acetonitrile. The separation was conducted at 0.4 mL/min with an initial hold until 1 min (elapsed time) at 2% B, a linear gradient to 40% B until 5 min, a ramp (0.1 min) to 90% B, hold for 1 min, then return ramp to 2% B. The stop time was 8 min. This gradient was used for both quantitative runs and the full-scan MS/MS confirmation of identity analysis. Under these conditions Cysteine eluted at 3.3 minutes, Cystine eluted at 4.2 minutes.

Sample preparation. Samples were diluted 10:1 in A buffer prior to injection. Standard concentrations are reported as the concentrations prior to this dilution.

Confirmation of identity. The singly-charged, protonated Cysteine ion (MH+) was selected for fragmentation in an injection of the standard solution at 1 μM concentration, and also for the sample injection. The full-scan fragment spectra were collected at a fragmentor voltage of 72 and collision energy of 15.

Mass spectrometer conditions for quantitation. The following transitions and conditions were used for Cysteine: 122 → 59, CE 22, Frag 72; 122 → 76 (qualifier), CE 10, Frag 72.

Cysteine quantitation. The equation from the standard curve from 0.1 μM to 10 μM was used to calculate the concentrations of Cys in all samples. These samples were analyzed in triplicate.

18. RNA interference

HS5 stromal cells were transfected with nonspecific, xCT RNA interference (RNAi; final concentrations, 20, 40, 100 nM) using Lipofectamine ^{TM} 2000 transfection according to the manufacturer's instructions (Invitrogen). The cells were then incubated in 24 well plates for 72 h prior to Western blot for xCT expression. The special designed Stealth

RANi^{TM} siRNA duplex oligo-ribonucleotides were purchased from Invitrogen. The RNA sequences for xCT were as follows: (siRNA set 1) sense, 5'-AGA UAA AUC AGC CCA GCA ACU GCC A-3', antisense, 5'-UCC CAG UUG CUG GGC UGA UUU AUC U-3'; (siRNA set 2) sense 5'-AUU AUG AGG AGU UCC ACC CAG ACU C-3', antisense, 5'-GAG UCU GGG UGG AAC UCC UCA UAA U-3'; (siRNA set 3) sense, 5'-UAA UGA GAA AUU UCC CAG UAG CCG C-3', antisense, 5'-UAA UGA GAA AUU UCC CAG UAG CCG C-3'; RNAi with the same GC content as siRNA pools was used as a negative control.

19. Statistical analyses

All experiments were done in CLL cells from at least three different patient samples, and with stromal cells from three separate culture flasks. Statistical significance was analyzed by the Student's t-test, and the p values of < 0.05 were considered statistically significant. Bar graphs and plots were generated using the Prizm software (GraphPad, San Diego, CA).

RESULTS

1. Bone marrow stromal cells protect CLL cells from spontaneous and drug-induced apoptosis.

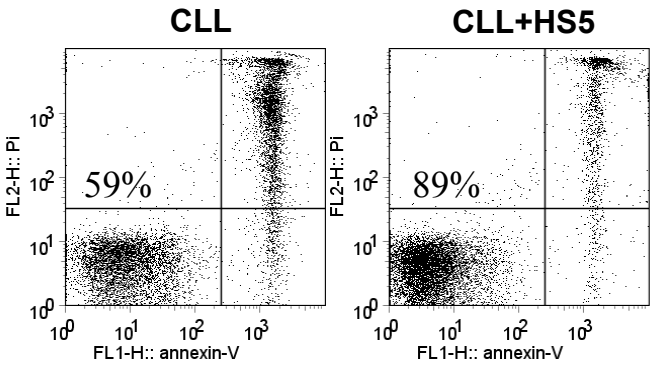
Chronic lymphocytic leukemia (CLL) B cells are characterized as a defect in apoptosis and exhibit prolonged survival *in vivo*, the accumulation of CLL cells eventually causes the bone marrow to fail and weakens the immune system. However, when recovered from peripheral blood from CLL patient and cultured *in vitro*, these anti-apoptotic CLL cells rapidly undergo spontaneous apoptosis, suggesting that the selective survival advantage enjoyed by CLL cells is not autonomous but likely manipulated by the *in vivo* microenvironment. To test this possibility, I employed an *in vitro* co-culture system including primary CLL cells isolated from peripheral blood of CLL patients, and a human bone marrow stromal cell line HS5, which was established by immortalizing of long-term bone marrow cultures (LTBMC) of human by human papolloma virus E6/E7 genes [109]. As shown in **Fig 4A**, CLL cells cultured alone *in vitro* exhibited substantial spontaneous apoptosis (41%) within 3 days, the presence of HS5 stromal in co-culture significantly enhanced CLL cell viability to 89%. I also tested the long term protective effect of stromal cells by continuously switching CLL cells to new flask with or without a layer of stromal cells to avoid the effect of nurse-like cell. Similar protective effect was also observed in a long-term (3 weeks) co-culture (**Fig 4B**). The ability of stromal cells to enhance CLL cell viability was consistently observed when the cells were cultured under ambient oxygen (21%) or under hypoxic conditions (2-5% O_2) in all 4 cases of CLL samples tested (**Fig 5)**, suggesting that this protective effect was the consequence of stromal-CLL cell interaction, not due to the artificial effect of the oxygen environment.

Altogether, these data suggest that bone marrow stromal cells protect CLL cells from spontaneous apoptosis in vitro both for short term and long term.

Fludarabine and oxaliplatin are currently used in clinical treatment of CLL. I then tested the effect of stromal cell on drug resistance of CLL cells. CLL cells isolated from peripheral blood of CLL patients were cultured with HS5 stromal cells for 1 day, and then treated with 20 μM F-ara-A (the active form of fludarabine) or 20 μM oxaliplatin for another 2 days. Apoptosis was detected by flow cytometry after staining with Annexin V/PI. As shown in **Fig 6A-B,** the presence of HS5 stromal cells significantly decreased cells death that occurred either spontaneously or induced by F-ara-A or oxaliplatin. Stromal-induced drug resistance in CLL cell was also confirmed in different co-culture systems with other two bone marrow stromal cell lines: StromaNKtert, which was established by immortalizing of long-term bone marrow cultures (LTBMC) of human by by human telomerase reverse transcriptase (hTERT) containing also exogene MFG-tsT-IRES-neo [110]; and KUSA-H1, a spontaneous immortalized cell line from long-term bone marrow cultures (LTBMC) of C3H/He mouse [111]. These two bone marrow stromal cell lines are kindly provided by Dr. Burger. Similar to HS5, both StromaNKtert and KUSA-H1 stromal cells protected CLL cell from spontaneous and drug-induced apoptosis (**Fig 7A**). Interestingly, compared to HS5 stromal cells that protected CLL cells for relative survival around 80% against drug treatment (**Fig 7A**), StromaNKtert and KUSA-H1 showed enhanced protection of CLL cells with around 90-100% relative survival (**Fig 7B**), indicating that certain protective mechanisms might be missing in HS5 stromal cells.

Figure 4. Bone marrow stromal cells promote CLL cell short-term and long-term survival in culture. (A) Comparison of viability of CLL cells isolated from a patient blood sample and cultured *in vitro* alone or with a layer of HS5 stromal cell for 3 days. Cell viability was assessed by Annexin-V/PI double staining on day 3. The numbers indicate the % of viable cells (annexin-V/PI double negative); **(B)** Comparison of long-term survival of CLL cells with or without HS5 stromal cells. CLL cells were transferred to new stromal layer when HS5 cells reached confluence. CLL cells alone were transferred to new flask in the same fashion to minimize the influence of nurse-like cells attached to the flask. Cell viability was assessed by Annexin-V/PI double staining. Figure displays phase-contrast photomicrophraphs that depict the morphologic appearance of CLL cells cultured alone or co-cultured with a layer of HS5 stromal cells. Cells were imaged in medium using a phase-contrast microscope with a 10X objective lens. Images were captured with a Nikon digital camera with the use of Camera Control Pro software (Nikon); when necessary, Adobe Photoshop 9.0 (Adobe Systems) was used for image processing. Photographs and flow cytometry analysis were performed on day 21.

A


CLL
CLL+HS5
59%
89%
FL2-H:: Pi
FL1-H:: annexin-V

B

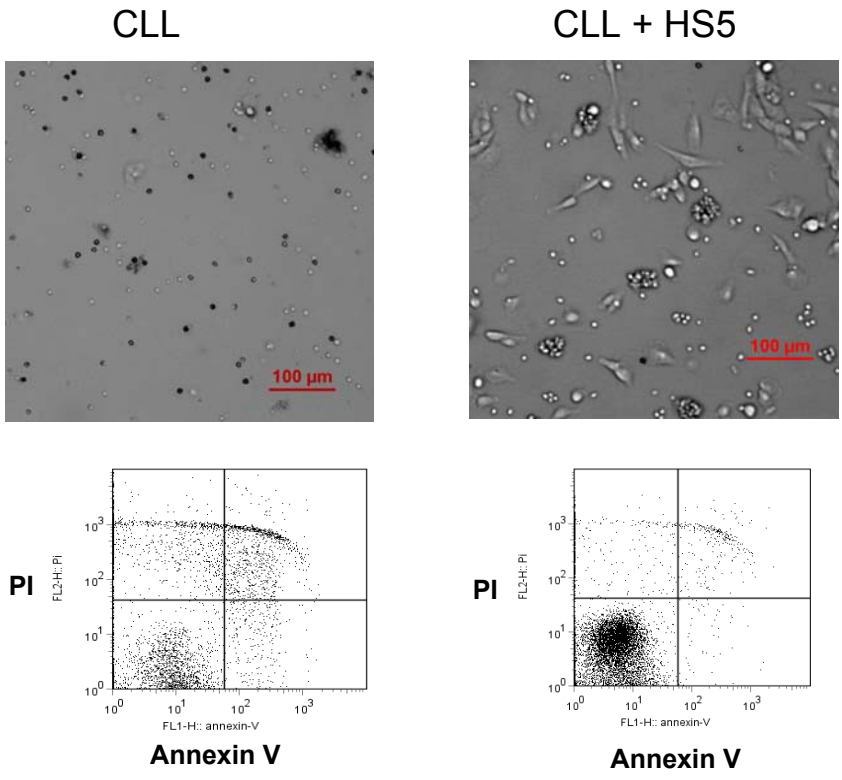
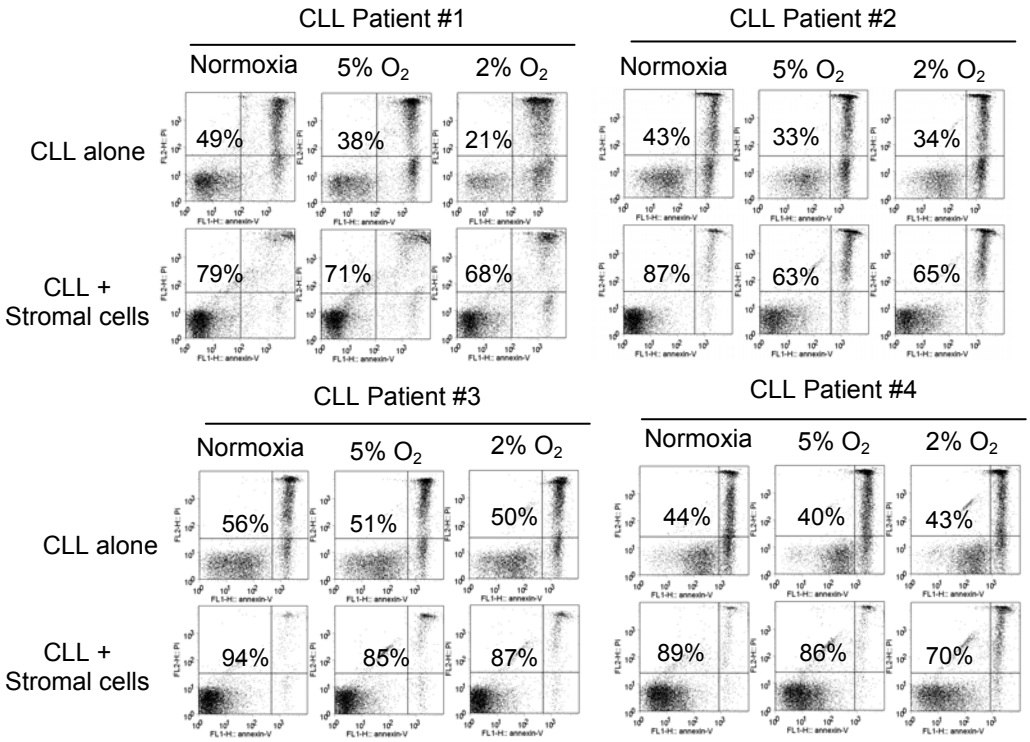

CLL
CLL + HS5
100 µm
100 µm
PI
PI
FL2-H:: PI
FL1-H:: annexin-V
Annexin V
Annexin V

Figure 5. Effect of oxygen levels on CLL cell viability in the presence and absence of bone marrow stromal cells. Primary CLL cells isolated from the blood samples of 4 CLL patients were incubated with bone marrow stromal cells (KUAS-H1) for 3 day, and cell viability was assessed by annexin-V/PI double staining. The bar graph showed the mean±SD of the 4 patient samples. *, $p<0.05$ (CLL alone vs co-culture with stromal cells).


CLL Patient #1
CLL Patient #2
CLL Patient #3
CLL Patient #4
Normoxia
5% O2
2% O2
CLL alone
CLL + Stromal cells
49%
38%
21%
43%
33%
34%
79%
71%
68%
87%
63%
65%
56%
51%
50%
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94%
85%
87%
89%
86%
70%

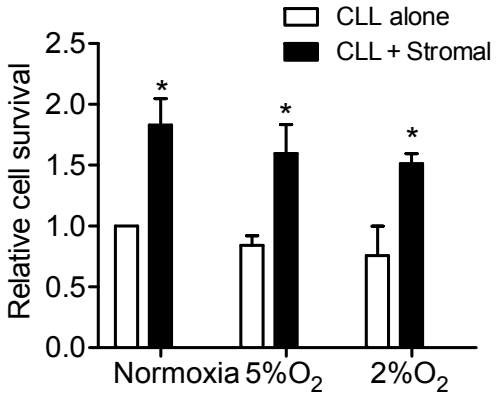
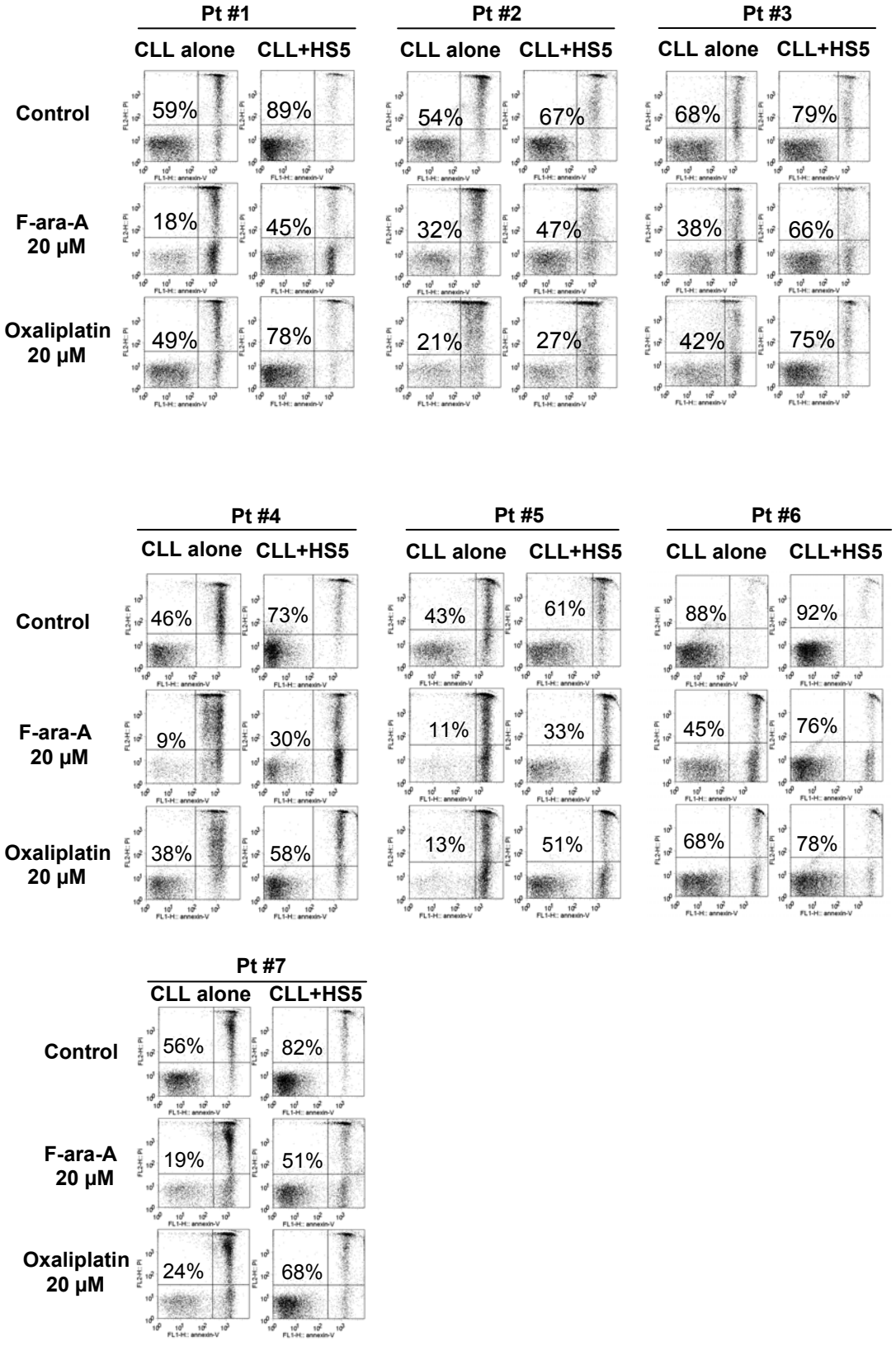

CLL alone
CLL + Stromal
Relative cell survival
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
Normoxia
5%O2
2%O2

Figure 6. Protection of CLL cells by bone marrow stromal HS5 cells in the presence or absence of F-ara-A (20 µM) or oxaliplatin (20 µM). (**A**) CLL cells were pre-cultured with HS5 cells for 24 h, followed by drug exposure for 48 h. Cell viability was measured by annexin-V/PI double staining. Representative dot plots of a CLL patient sample are shown; the numbers indicates the % of viable cells (annexin-V/PI double negative). (**B**) The mean ± SEM of 7 separate experiments using patient samples. *, $p<0.05$ (CLL cultured alone vs co-cultured with stromal cells).

A


Pt #1
CLL alone
CLL+HS5
Control
F-ara-A 20 µM
Oxaliplatin 20 µM
59%
89%
18%
45%
49%
78%
Pt #2
54%
67%
32%
47%
21%
27%
Pt #3
68%
79%
38%
66%
42%
75%
Pt #4
46%
73%
9%
30%
38%
58%
Pt #5
43%
61%
11%
33%
13%
51%
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56%
82%
19%
51%
24%
68%

B

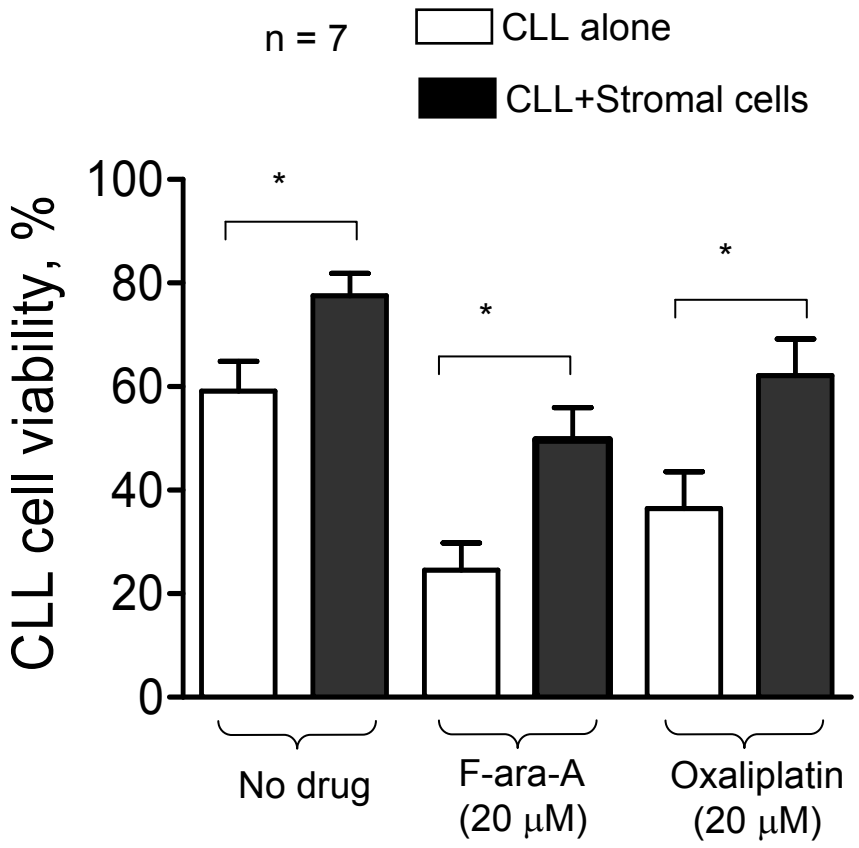
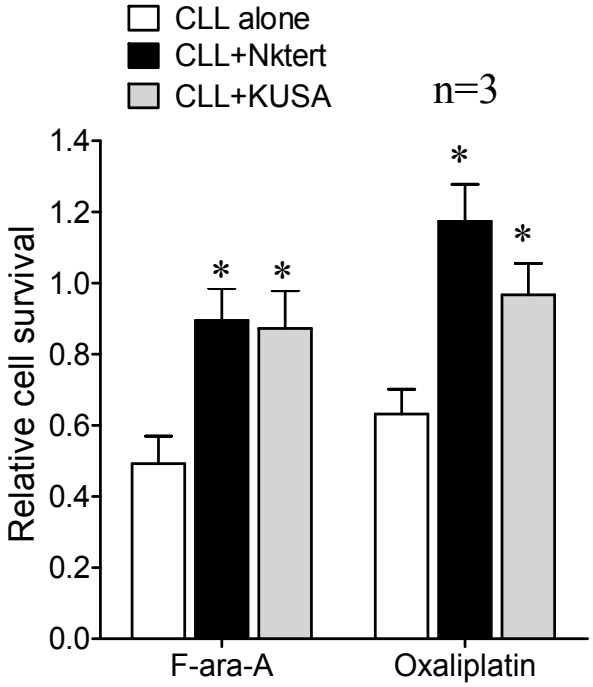
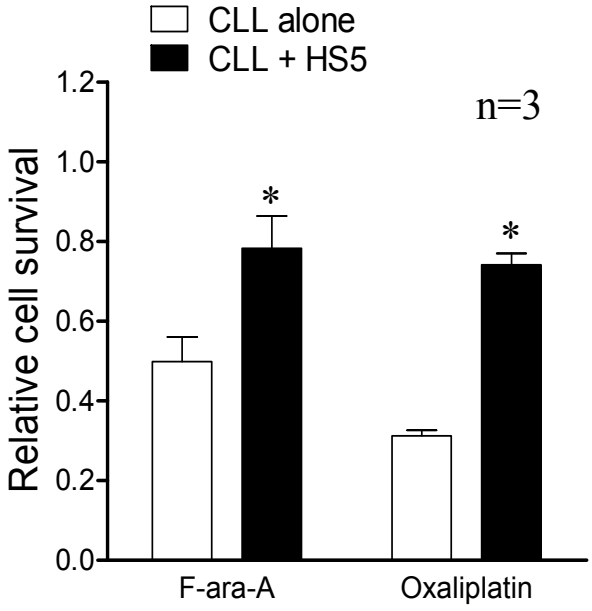

n = 7
CLL alone
CLL+Stromal cells
CLL cell viability, %
100
80
60
40
20
0
*
*
*
No drug
F-ara-A
(20 μM)
Oxaliplatin
(20 μM)

Figure 7. Protection of CLL cells by bone marrow stromal cells StromalNKtert and KUSA-H1 (A), and HS5 (B) in the presence or absence of F-ara-A (20 μM) or oxaliplatin (20 μM). CLL cells were pre-cultured with HS5/StromaNKtert/KUSA-H1 cells for 24 h, followed by drug exposure for 48 h. Cell viability was measured by annexin-V/PI double staining. The mean ± SEM of 3 separate experiments using patient samples were shown. *, $p<0.05$ (CLL cultured alone vs co-cultured with stromal cells).

A


CLL alone
CLL+Nktert
CLL+KUSA
n=3
Relative cell survival
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
*
F-ara-A
Oxaliplatin

B


CLL alone
CLL + HS5
n=3
Relative cell survival
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
F-ara-A
Oxaliplatin

2. Bone marrow stromal cells promote GSH synthesis in CLL cells and relieve their ROS stress

Previous studies showed that CLL cells are under intrinsic oxidative stress [49, 113] and exhibit high spontaneous apoptosis with rapid GSH depletion *in vitro* but have a prolonged survival time *in vivo* [98, 114]. These observations prompted me to test the hypothesis that the stromal microenvironment *in vivo* might promote GSH synthesis in CLL cells and enhance their ability to keep redox balance and remain viable. When the cellular GSH in CLL cells cultured alone or co-cultured with HS5 stromal cells was measured, I observed a striking difference. CLL cells cultured alone showed a time-dependent decrease in cellular GSH, whereas GSH in CLL cells co-cultured with HS5 were maintained at a high level (**Fig 8**). Comparison of GSH levels in 35 CLL patient samples cultured for 3 days with or without HS5 stromal cells showed that the GSH levels were about 7-fold higher in CLL cells co-cultured with the bone marrow stromal cells (**Fig 9A**). Detail data analysis revealed that 33 out of the 35 CLL patient samples exhibited more than 100% increase in cellular GSH levels in the presence of HS5, with the majority of cellular GSH in the range of 1.5-4 nmole/10^7 cells (**Fig 9B**). In the absence of stromal cells, the majority of CLL cells had GSH contents of less than 0.5 nmole/10^7 cells on day 3, and 10 out of the 35 samples had less than 0.2nmole GSH/10^7cells.

Because GSH is a key antioxidant in the cells, I tested whether the presence of HS5 stromal cells could relieve the oxidative stress in CLL cells. Cellular ROS levels and thiol contents (as an indication of cellular GSH levels) were measured by flow cytometry using the fluorescence probes DCF-DA and CMFDA, respectively, as described

previously [52, 115, 116]. I showed that CLL cells isolated from 20 different CLL patient co-cultured with HS5 stromal cells had a significantly lower ROS (**Fig 10A**) and a higher cellular thiol content (**Fig 10B**) at day 3. This observation is consistent with the increased GSH content in CLL cells with a layer of stromal cells. Moreover, this change in redox status rendered the CLL cells highly resistant to exogenous oxidative stress imposed by H_2O_2. As shown in **Fig 11**, exposure of primary CLL cells from 3 patient samples to 100 μM H_2O_2 caused massive cell death (70-90%) when the leukemia cells were cultured alone, while the presence of HS5 cells consistently protected CLL cells from the cytotoxic effect of exogenous H_2O_2 in all three cases.

To evaluate if the ability of bone marrow stromal cells to increase GSH contents and reduce ROS levels in CLL cells was a general phenomenon or only specific to HS5 cells, I tested two other bone marrow stromal cell lines StromaNKtert and KUSA-H1 for their ability to enhance GSH synthesis and reduce oxidative stress in CLL cells in the co-culture system. As shown in **Fig 12,** all these bone marrow stromal cells were able to significantly increase GSH in all cases of primary CLL cells from 6 different CLL patients. Consistently, flow cytometry analysis of cellular ROS and thiols content also showed that all three bone marrow stromal cells decreased ROS contents and enhanced the cellular thiol levels in CLL cells (**Figs 13A-B**).

Figure 8. Bone marrow stromal cells maintain GSH level in CLL cell. CLL cells isolated from 3 different patients were single- or co-cultured with HS5 stromal cells. CLL cells with or without a layer of stromal cells were collected at 6, 12, 24, 48h. GSH levels in CLL cells at various time points were analyzed altogether. The chart shows the time course of GSH contents in CLL cells cultured alone or with HS5 stromal cells.

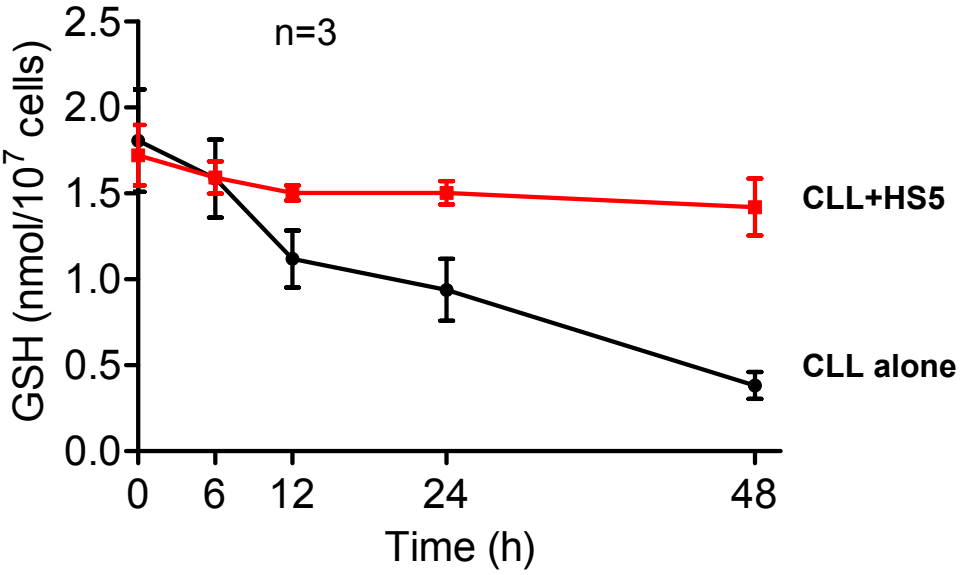
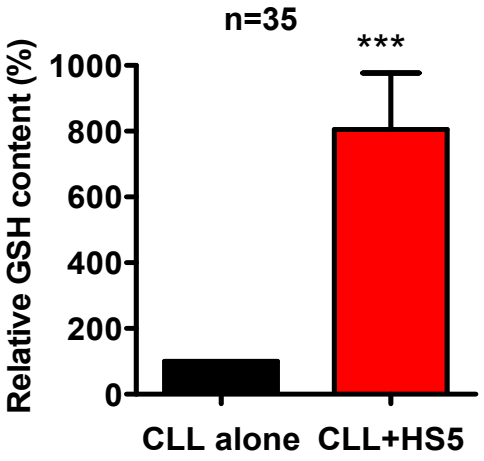

n=3
GSH (nmol/10^7 cells)
2.5
2.0
1.5
1.0
0.5
0.0
0
6
12
24
48
Time (h)
CLL+HS5
CLL alone

Figure 9. Enhancement of GSH synthesis in CLL cells by HS5 stromal cells. (A) Comparison of cellular GSH levels after 72 h in culture alone or with HS5 stromal cells. The bar graph shows mean ±SEM of 35 different CLL samples (***, $p<0.001$). **(B)** Each bar shows the mean (±SEM) of the GSH concentration in each CLL sample measured in triplicates (n= 35 different CLL samples; the "*" symbols for patients #9, #18, #30, and #32 indicate that GSH was undetectable in these CLL samples (cultured alone).

A


n=35
***
Relative GSH content (%)
1000
800
600
400
200
0
CLL alone
CLL+HS5

B

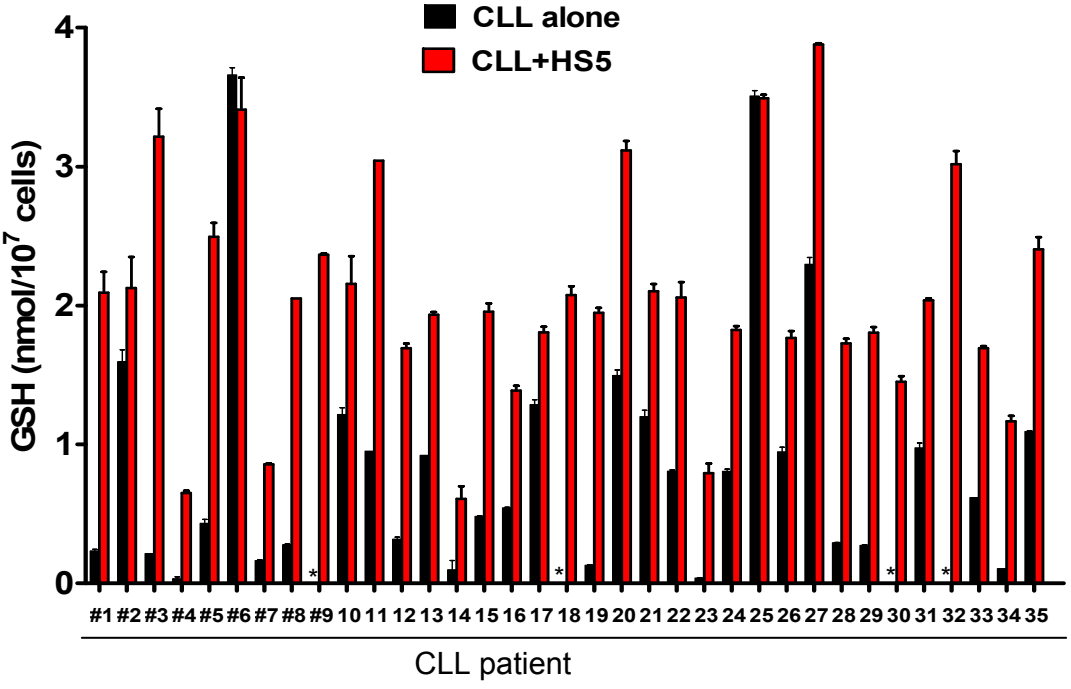
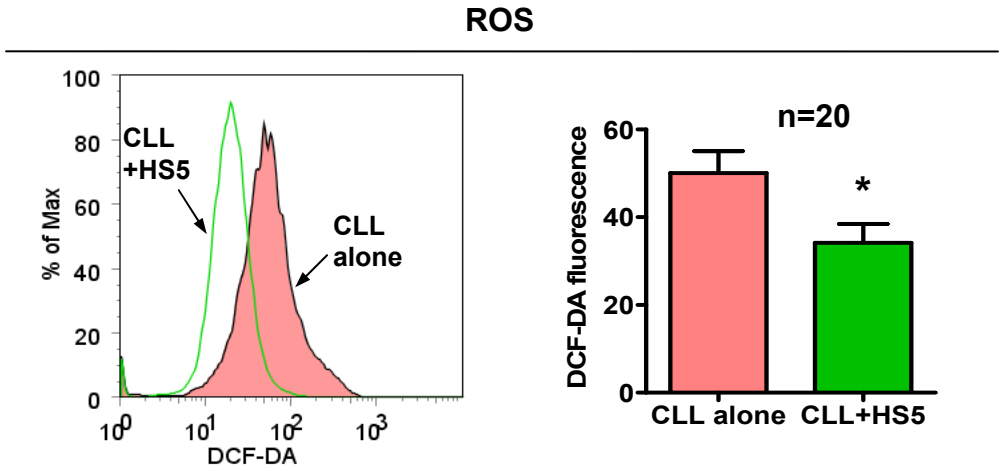

CLL alone
CLL+HS5
GSH (nmol/10^7 cells)
4
3
2
1
0
#1 #2 #3 #4 #5 #6 #7 #8 #9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35
CLL patient

Figure 10. Alteration of redox status of CLL cells in the presence of bone marrow stromal cell. Determination of cellular ROS and thiol contents in CLL cells cultured alone or with HS5 stromal cells. Fluorescent probes DCF-DA and CMFDA were used to detect cellular ROS levels **(A)** and total thiol levels **(B)** respectively by flow cytometric analysis. Representative histograms and quantitative comparison of mean values from 20 different CLL samples are shown (*, $p< 0.05$; **, $p<0.01$).

A


ROS
100
80
60
40
20
0
% of Max
CLL
+HS5
CLL
alone
10^0
10^1
10^2
10^3
DCF-DA
n=20
*
DCF-DA fluorescence
60
40
20
0
CLL alone
CLL+HS5

B

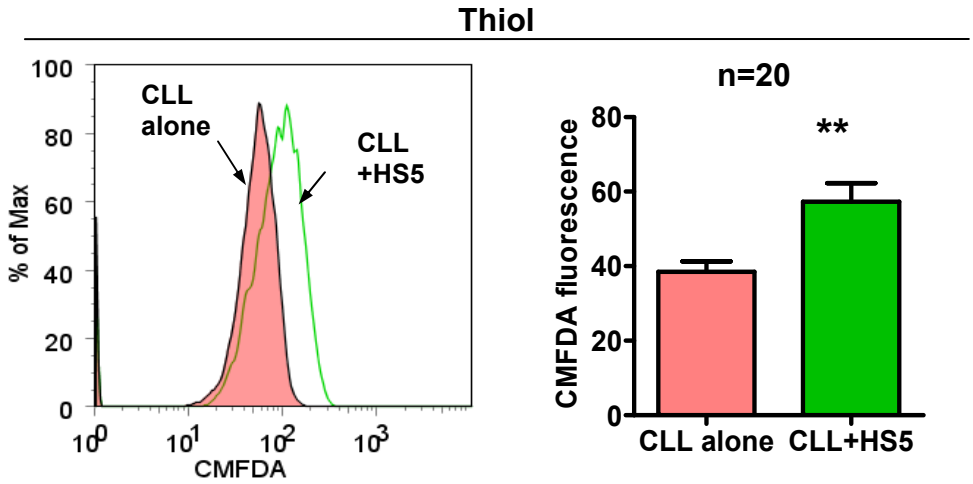

Thiol
100
80
60
40
20
0
% of Max
CLL
alone
CLL
+HS5
10^0
10^1
10^2
10^3
CMFDA
n=20
**
CMFDA fluorescence
80
60
40
20
0
CLL alone
CLL+HS5

Figure 11. Bone marrow stromal cells protect CLL cells from spontaneous apoptosis and cell death induced by H_2O_2 (100 μM). CLL cells isolated from 3 different patients were single- or co-cultured with HS5 stromal cells for 2 days and treated with 100 μM H_2O_2 for overnight. Cell viability was measured by annexin V-PI staining. The number in each dot blot indicates the average % of viable cells (annexin-V/PI double negative) from experiments using 3 different CLL samples.

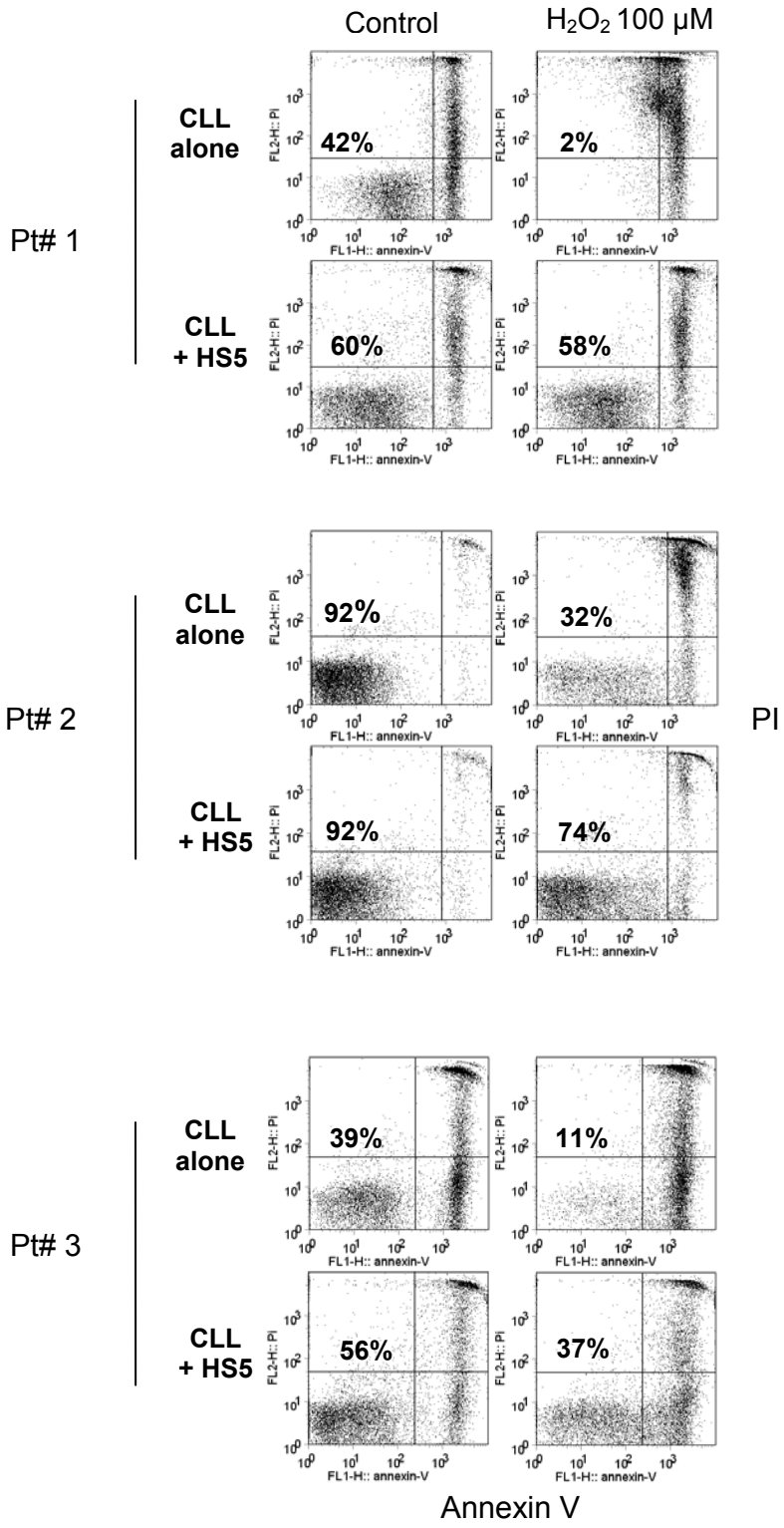

Control
H2O2 100 µM
Pt# 1
CLL alone
CLL + HS5
42%
2%
60%
58%
Pt# 2
CLL alone
CLL + HS5
92%
32%
92%
74%
Pt# 3
CLL alone
CLL + HS5
39%
11%
56%
37%
FL2-H:: PI
FL1-H:: annexin-V
PI
Annexin V

Figure 12. Enhancement of GSH synthesis in CLL cells by bone marrow stromal cells. CLL cell were isolated from peripheral blood of patients and cultured alone or with a layer of different bone marrow stromal cells (HS5, StromaNKtert, KUSA-H1). GSH levels in CLL cells were analyzed at day 3. Each bar shows the mean±SEM of the GSH contents in 6 different CLL samples measured in triplicates.

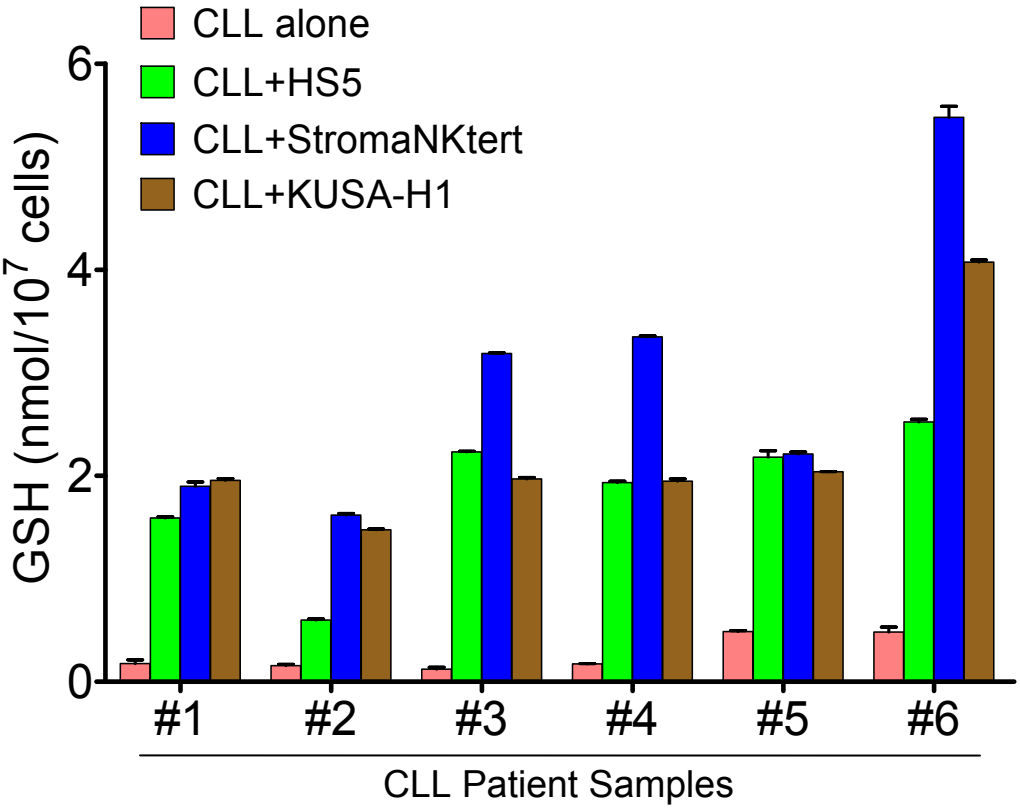
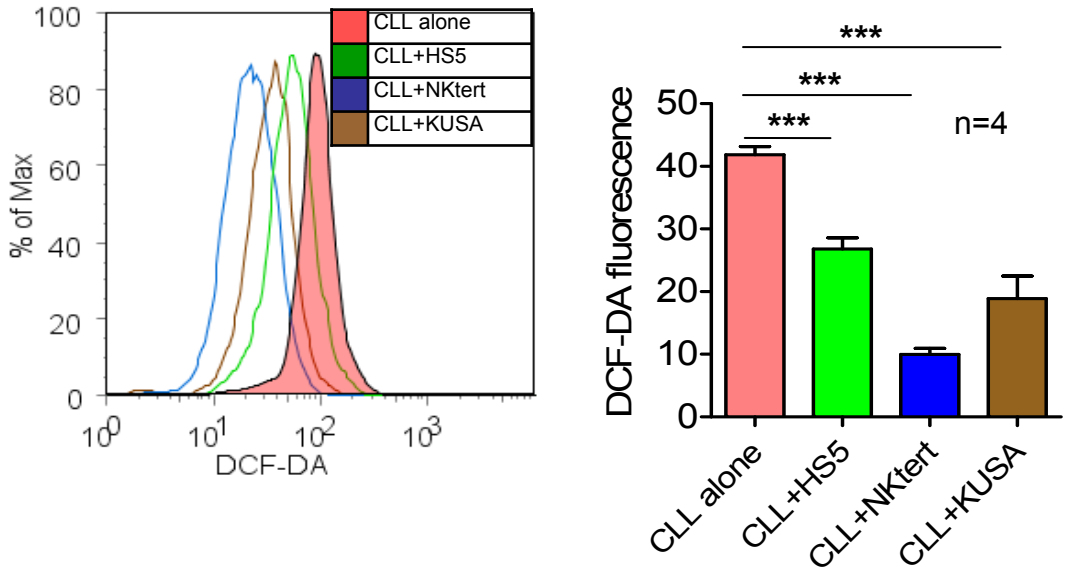

CLL alone
CLL+HS5
CLL+StromaNKtert
CLL+KUSA-H1
GSH (nmol/10^7 cells)
0
2
4
6
#1
#2
#3
#4
#5
#6
CLL Patient Samples

Figure 13. Bone marrow stromal cells decrease cellular ROS and increase total thiol in CLL cells. (A) Comparison of cellular ROS in CLL cells after a 3-day culture alone or with a layer of HS5, StromaNKtert, and KUSA-H1 stromal cells. Cellular ROS were detected by flow cytometry using 1 μM DCF-DA. Representative histograms and the means ±SEM of 4 separate experiments with different CLL samples are shown (***, $p<0.001$). (**B**) Comparison of total cellular thiols in CLL cells after a 3-day culture alone or with HS5, StromaNKtert, and KUSA-H1 stromal cells. Total cellular thiols were detected by flow cytometry using 0.5 μM CMFDA as a probe. Representative histograms (left panel) and the means ± SEM of 4 separate experiments with 4 different CLL samples are shown (*, $p<0.05$; **, $p<0.01$).

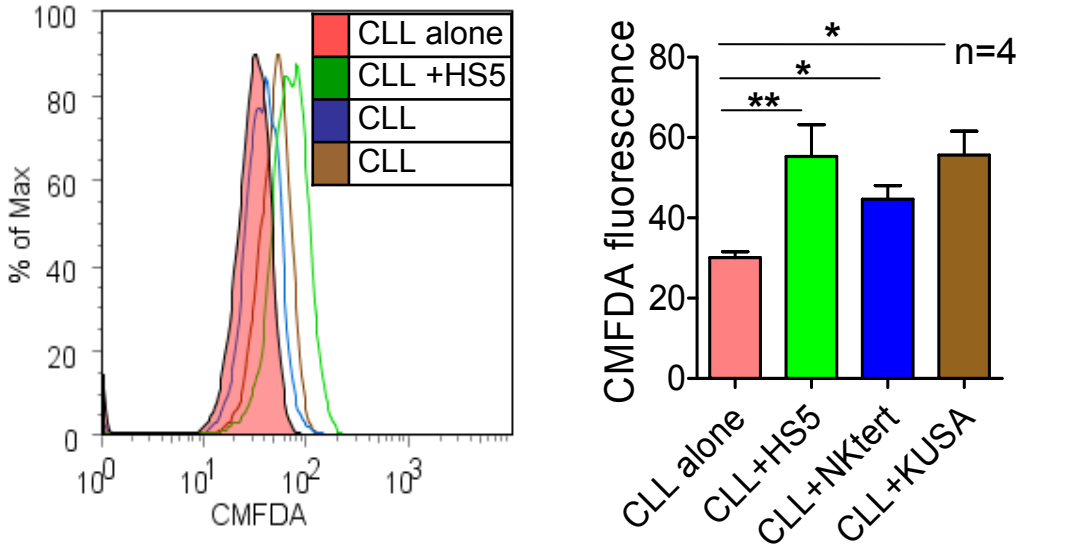
A

ROS



B

Thiols



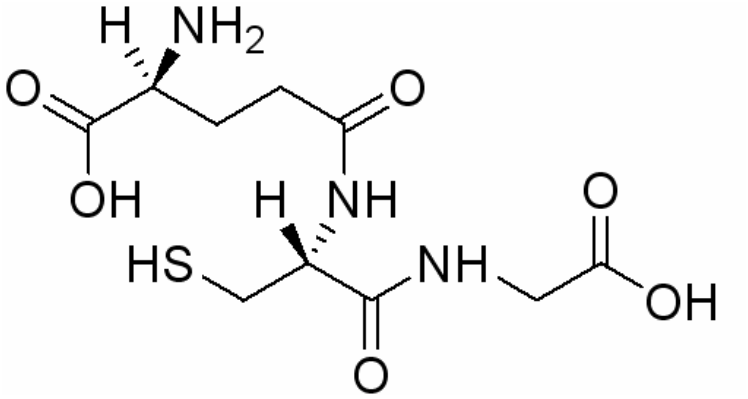
3. GSH plays a key role in mediating stromal protection of CLL cells from spontaneous- and drug-induced apoptosis.

After showing that bone marrow stromal cells maintained GSH level in CLL cells, the role of GSH in mediating stromal protection of CLL cells from apoptosis was then tested in the co-culture system with or without drug incubation. Because GSH is the most abundant antioxidant involved in cell survival and drug resistance [95, 116], I then tested whether the high level of GSH in CLL cells conferred by HS5 stromal cells was important for their survival. CLL cells in suspension culture were incubated in the following 3 conditions: (1) CLL cells alone in standard RPMI media, (2) with a layer of HS5 cells, (3) with glutathione (GSH) or its precursor N-acetylcysteine (NAC) without stromal cells. The structures of GSH and NAC are shown in **Fig 14A-B**. Cell death was monitored at various time intervals by annexin-V/PI staining. As shown in **Fig 15A**, CLL cells cultured alone exhibited a time-dependent loss of viability, with spontaneous apoptosis reaching as high as 70% on day 10. In contrast, co-culture with HS5 stromal cells significantly enhanced CLL cell survival with only a loss of 20% viability on day 10. Importantly, supplement of the NAC also effectively prevented apoptosis in CLL cells in the absence of stromal cells (**Fig 15A**), suggesting that the increase in GSH by NAC supplement was sufficient to enhance cell survival. NAC also protected CLL cells from oxaliplatin-induced cytotoxicity (**Fig 15B**). The ability of GSH to promote CLL cell viability was further demonstrated in a separate experiment using CLL cells from 3 independent patient blood samples, which showed massive spontaneous apoptosis 14 days after being cultured *in vitro* alone, but remained largely viable when the culture medium was supplemented with 2 mM GSH without stromal cells (**Fig 16**). Analysis of

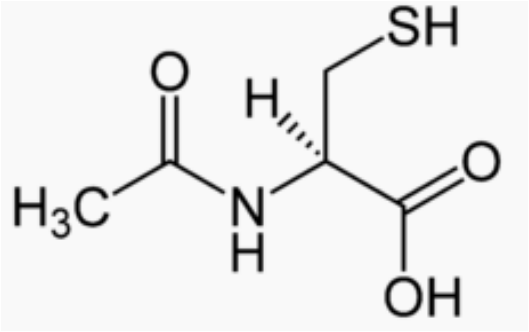
cellular GSH showed that CLL cells cultured alone without NAC/GSH supplements lost 80% of their cellular GSH in 3 days and lost almost all GSH in 7 days, while the presence of NAC (1 mM) or GSH (2 mM) enabled CLL cells to maintain cellular GSH in the absence of stromal cells (**Fig 17**). The effect of different concentrations of NAC and GSH on long term survival of CLL cells was also tested. As shown in **Fig 18A-B**, 20 μM NAC or GSH failed to protect CLL cells and 200 μM NAC or GSH have some limited protective effect in CLL cells within 14 days. However, only higher concentrations of NAC (2mM) or GSH (2mM) exerted strong protection in CLL cells within 18 days, indicating that only nonphysical concentrations of GSH could protect CLL cells from spontaneous apoptosis for long term. The important role of GSH in mediating stromal protection of CLL cells was further demonstrated by induction of CLL cell death in the presence of stromal cells through pharmacological depletion of GSH using β-phenylethyl isothiocyanate (PEITC), a natural compound capable of rapidly depriving cellular glutathione [52, 117]. The structure of PEITC is shown in **Fig 14C**. As shown in **Fig 19**, PEITC (5 μM) significantly decreased the GSH content in CLL cells co-cultured with HS5 stromal cells, which would otherwise cause a significant increase of GSH in CLL cells without PEITC. Depletion of GSH by 5 μM PEITC was toxic to CLL cells and significantly enhanced the cytotoxicity of F-ara-A or oxaliplatin in the presence of HS5 (**Fig 20**). In the absence of PEITC, HS5 stromal cells enhanced the viability of CLL cells exposed to F-ara-A or oxaliplatin, consistent with **Fig 6**.

Figure 14. Structure of glutathione (GSH) (A), N-acetylcysteine (NAC) (B), and β-phenylethyl isothiocyanate (PEITC) (C).

A



B



C

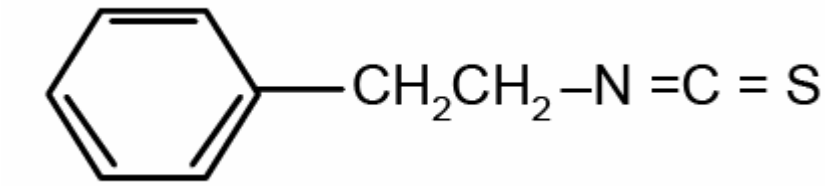
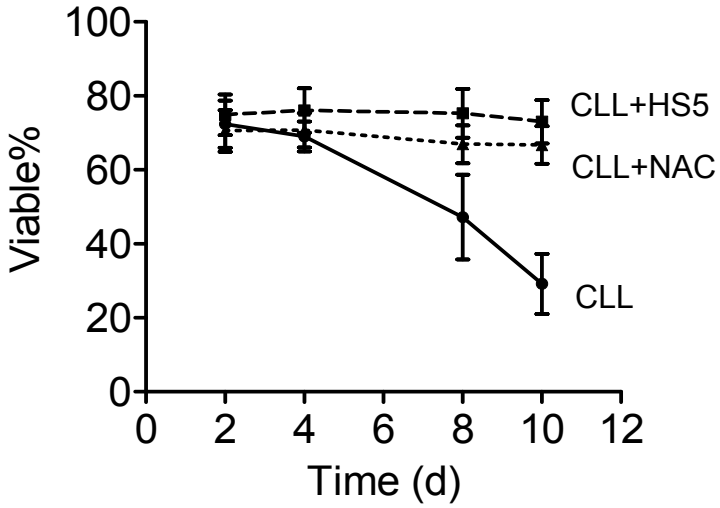


Figure 15. N-acetylcysteine (NAC) and bone marrow stromal cells protect CLL cells from spontaneous and drug-induced apoptosis. (A) Increase of CLL cell viability by HS5 stromal cells or exogenous NAC. CLL cells were cultured alone, with HS5 cells, or with 1mM NAC for the indicated times. Cell viability was measured by flow cytometry analysis. n= 3 different CLL samples for each conditions. **(B)** Comparison of the ability of N-acetylcysteine and bone marrow stromal cells to protect CLL cells from spontaneous and drug-induced apoptosis. CLL cells were cultured alone, with 1 mM N-acetylcysteine (NAC, a metabolic precursor of GSH), or with bone marrow stromal cells (HS5) in the presence or absence of oxaliplatin (20 μM) for 48h. Cell viability was measured by annexin-V/PI staining. The number in each dot blot indicates % of viable cells (annexin-V/PI double negative).

A


100
80
60
40
20
0
Viable%
0
2
4
6
8
10
12
Time (d)
CLL+HS5
CLL+NAC
CLL

B

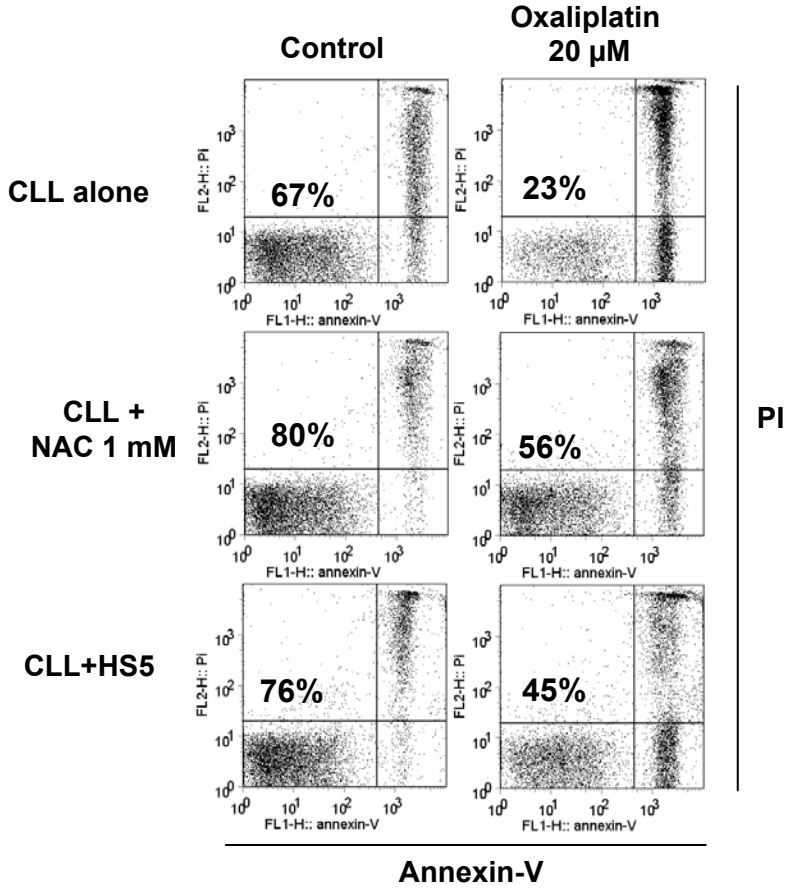

Control
Oxaliplatin
20 µM
CLL alone
CLL +
NAC 1 mM
CLL+HS5
67%
23%
80%
56%
76%
45%
FL2-H:: PI
FL1-H:: annexin-V
PI
Annexin-V

Figure 16. Effective protection of CLL cells by exogenous GSH in the culture medium without stromal cells. CLL cells were cultured in medium with or without GSH (2mM) for 14 d, and cell viability was measured by flow cytometry after staining with Annexin V and PI.

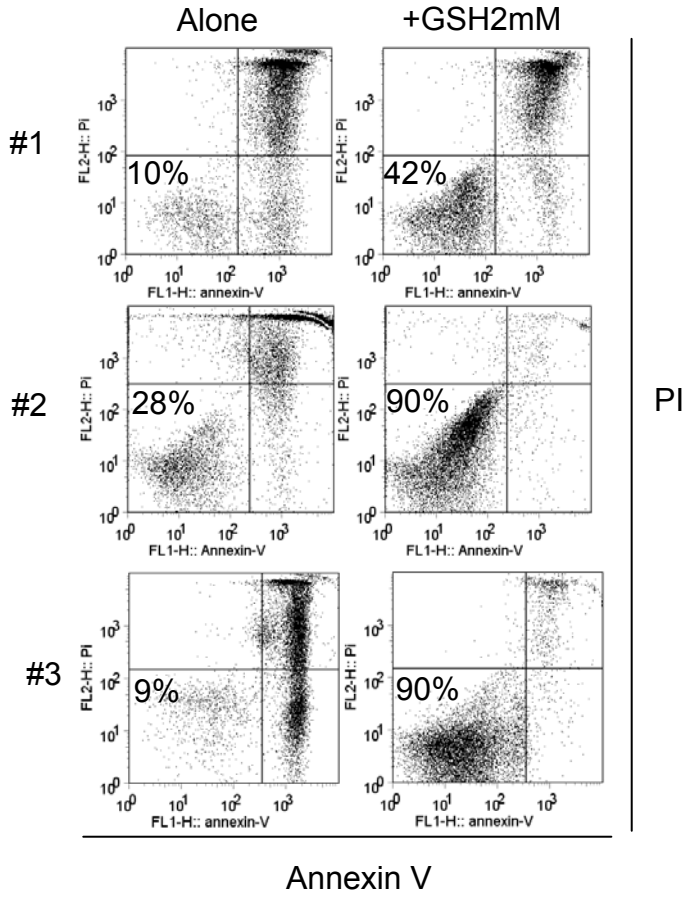
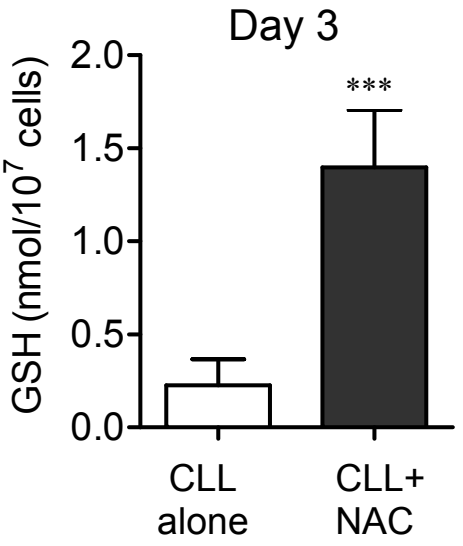

Alone
+GSH2mM
#1
#2
#3
10%
42%
28%
90%
9%
90%
FL2-H:: PI
FL1-H:: annexin-V
FL1-H:: Annexin-V
PI
Annexin V

Figure 17. Effect of NAC (1 mM, 3 days) or GSH (2 mM, 7 days) on GSH levels in CLL cells cultured without stromal cells. CLL cells were incubated with 1 mM NAC for 3 days **(A)** or 2 mM GSH for 7 days **(B)** in the absence of stromal cells. At the end of the incubation, cell extracts were analyzed for GSH. CLL cells cultured without NAC and GSH were used as the control for comparison. Each bar represents mean SD of three separate measurements.

A


Day 3
GSH (nmol/10^7 cells)
2.0
1.5
1.0
0.5
0.0
***
CLL alone
CLL+ NAC

B

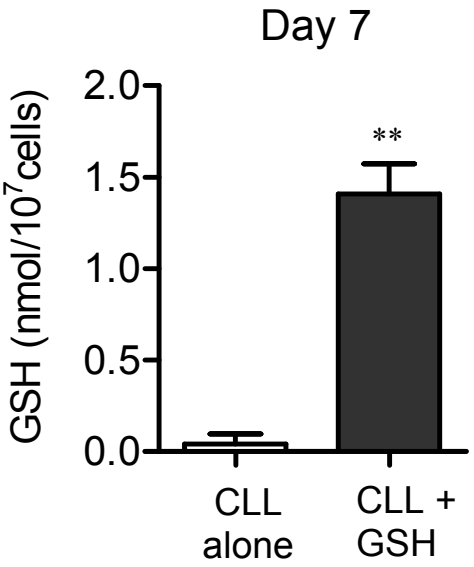
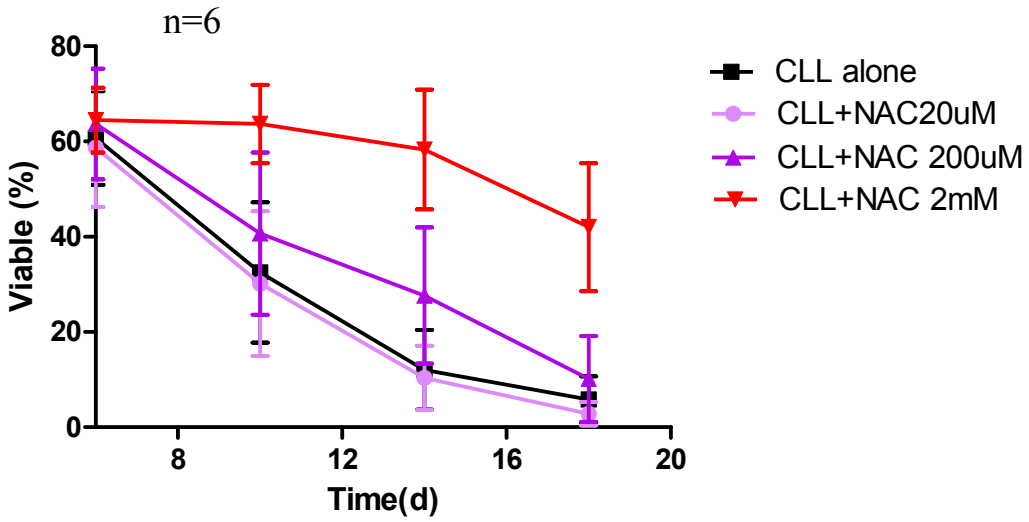

Day 7
GSH (nmol/10^7cells)
2.0
1.5
1.0
0.5
0.0
**
CLL alone
CLL + GSH

Figure 18. The effect of various doses of N-acetylcysteine (NAC) and glutathione (GSH) on the survival of CLL cells. CLL cells isolated from peripheral blood of CLL patients were cultured alone or with 20 μM, 200 μM, 2mM NAC (A), or with 20 μM, 200 μM, 2mM NAC (B) for indicated times. Cell viability was measured by flow cytometry analysis. n=3 different CLL samples fro each conditions.

A


n=6
Viable (%)
Time(d)
CLL alone
CLL+NAC20uM
CLL+NAC 200uM
CLL+NAC 2mM

B

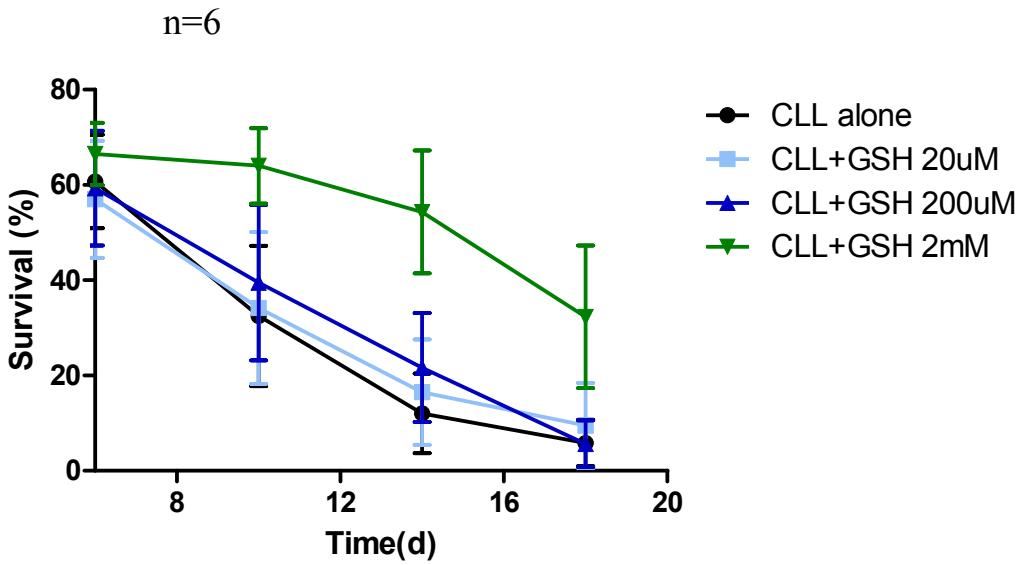

n=6
Survival (%)
Time(d)
CLL alone
CLL+GSH 20uM
CLL+GSH 200uM
CLL+GSH 2mM

Figure 19. β-phenylethyl isothiocyanate (PEITC) depletes cellular thiols in CLL cells cultured alone or with HS5 stromal cells. CLL cells were cultured alone or with a layer of HS5 stromal cells for 3 days and treated with 5 μM PETIC for 5h. Fluorescent probes CMFDA was used to detect cellular total thiol level by flow cytometric analysis.

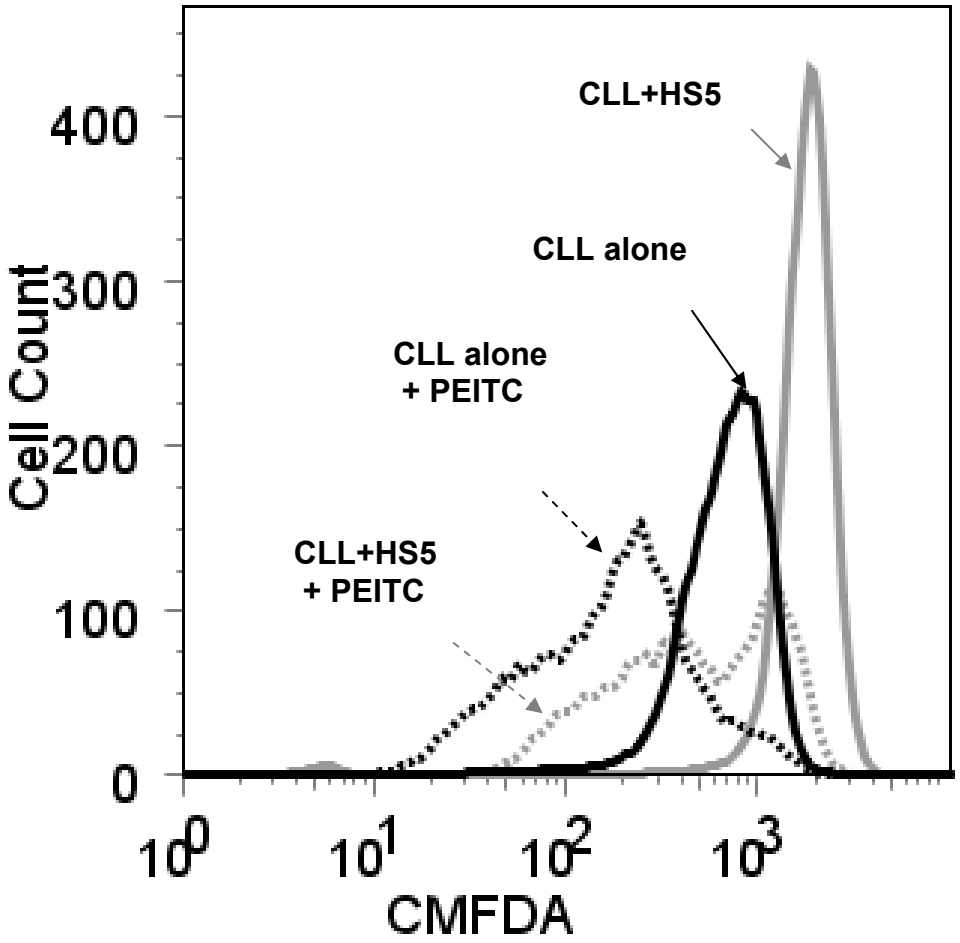
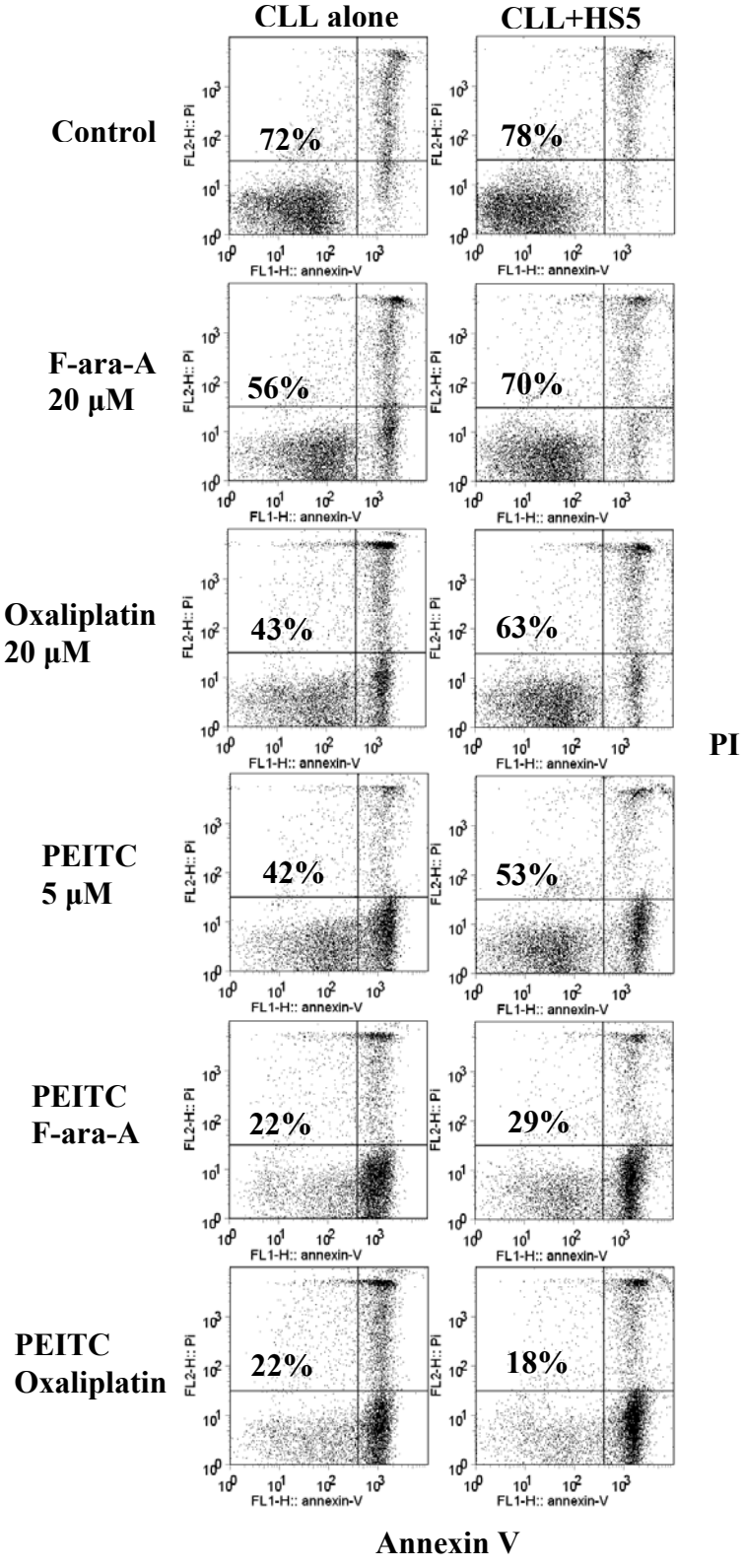

CLL+HS5
CLL alone
CLL alone
+ PEITC
CLL+HS5
+ PEITC
Cell Count
400
300
200
100
0
10^0
10^1
10^2
10^3
CMFDA

Figure 20. Enhancement of cytotoxicity of F-ara-A and oxaliplatin by PETIC in the presence of HS5 stromal cells. CLL cells were cultured alone or with a layer of HS5 stromal cells for 1 day, and then treated with 20 μM F-ara-A, 20 μM oxaliplatin for another 48 hours, 5 μM PEITC for 5 hours, or their combination as indicated. Cell viability was measured by flow cytometry analysis after staining with annexin V/PI. The representative dot plot is shown. The number in each dot blot indicates % of viable cells (annexin V/PI double negative).


CLL alone
CLL+HS5
Control
72%
78%
F-ara-A
20 µM
56%
70%
Oxaliplatin
20 µM
43%
63%
PEITC
5 µM
42%
53%
PEITC
F-ara-A
22%
29%
PEITC
Oxaliplatin
22%
18%
FL2-H:: PI
FL1-H:: annexin-V
PI
Annexin V

4. Soluble factor derived from bone marrow stromal cells enhance GSH synthesis in CLL cells and promotes cell survival.

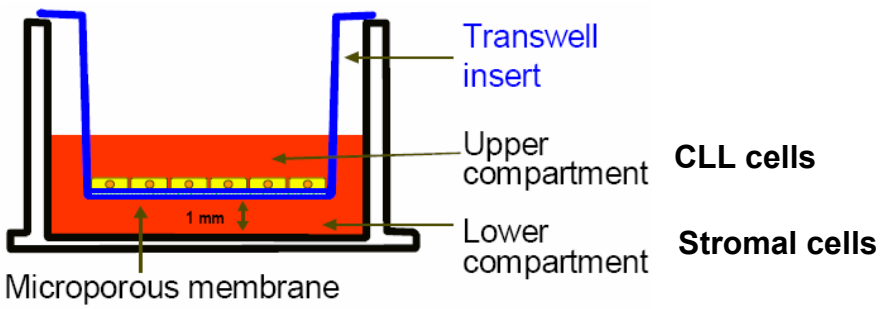
Since GSH seemed to play a major role in mediating stromal protection of CLL cells, I next investigated how the bone marrow stromal cells maintain GSH at a high level in CLL cells. First, I used a transwell co-culture system in which CLL cells were cultured within an insert chamber with a microporous membrane that prevented the direct contact between CLL cells and the stromal cells in the outer chamber, but allowed the exchange of soluble factors between the two compartments (**Fig 21A**). As shown in **Fig 21B,** co-culture of CLL cells with HS5 stromal cells in the transwell system showed a significant protective effect against cell death induced by F-ara-A (20 μM), oxaliplatin (20 μM), or H_2O_2 (100 μM). This protective effect was similar to that observed in direct co-culture without a membrane separation of the two cell types, suggesting that the protective effect was largely mediated by the soluble factors in the medium. Such protective effect was consistently observed using two other bone marrow stromal cell lines (StromaNKtert and KUSA-H1) in similar co-culture settings (**Fig 22**). Interestingly, with StromaNktert and KUSA-H1, the protective effect in the direct co-culture without membrane separation appeared greater than that observed in the transwell system, suggesting that the direct contact between stromal and CLL cells also contributed to the overall protective effect (**Fig 22**). Previous I showed that the overall protective effect of StromaNKtert and KUSA-H1 were higher than HS5 stromal cells (**Fig 7**). It is likely that cell contact mediated protection is missing in co-culture system with HS5 stromal cells but exist in those with StromaNKtert or KUSA-H1. The morphology of CLL cells cultured with different stromal cells was quite different. When co-cultured with HS5 stromal cells, CLL

cells easily formed clusters and flowed in the medium (**Fig 23**). However, with a layer of StromaNKtert or KUSA-H1, CLL cells attached to the stromal cell layer without forming clusters (**Fig 23**). There are two possible reasons for no cell contact between CLL cells and HS5 stromal cells. Firstly, HS5 stromal cells might miss certain adhesion molecules on the cell surface; secondly, some soluble forms of adhesion molecules might be secreted by HS5 cells that bind to CLL cells thus block the interaction with cell surface forms of adhesion molecules, such as sICAM-1, which was detected by cytokine array in the HS5 cell-conditioned medium (**Fig 25**).

The ability of soluble stromal factor to protect CLL cells was further confirmed using the conditioned medium from the HS5 stromal cell culture. The cell-free conditioned medium (CM) increased GSH in CLL cells and enhanced their survival in culture without stromal cells (**Fig 24**).

Figure 21. Protection of CLL cells against spontaneous and drug-induced apoptosis by HS5 stromal cells. (**A**) Comparison of drug-induced loss of cell viability in CLL cells cultured alone or with a layer of HS5 stromal cells in the presence or absence of a micropore membrane (filter) (**B**), which separated CLL cells from the stromal cells but allow the diffusion of soluble factors. After pre-incubation, the cells were treated with F-ara-A (20 μM, 48 h), oxaliplatin (20 μM, 48 h), or H_2O_2 (100 μM, 24 h). Cell viability was assessed by annexin-V/PI staining. * indicates $p<0.05$ compared to the sample without stromal cells.

A


Transwell
insert
Upper
compartment
CLL cells
1 mm
Lower
compartment
Stromal cells
Microporous membrane

B

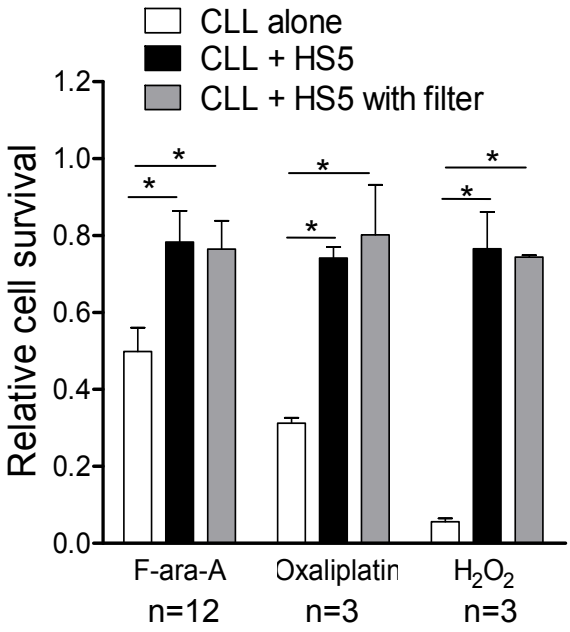

CLL alone
CLL + HS5
CLL + HS5 with filter
Relative cell survival
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
F-ara-A
Oxaliplatin
H2O2
n=12
n=3
n=3

Figure 22. Protection of CLL cells against spontaneous and drug-induced apoptosis by StromaNKtert and KUSA-H1 stromal cells. Comparison of drug-induced loss of cell viability in CLL cells cultured alone or with a layer of StramaNKtert or KUSA-H1 cells in the presence or absence of a micropore membrane (filter), which separated CLL cells from the stromal cells but allow the diffusion of soluble factors. After pre-incubation, the cells were treated with F-ara-A (20 mM, 48 h), oxaliplatin (20 mM, 48 h), or H_2O_2 (100 mM, 24 h). Cell viability was assessed by annexin-V/PI staining. * indicates $p<0.05$ compared to the sample without stromal cells.

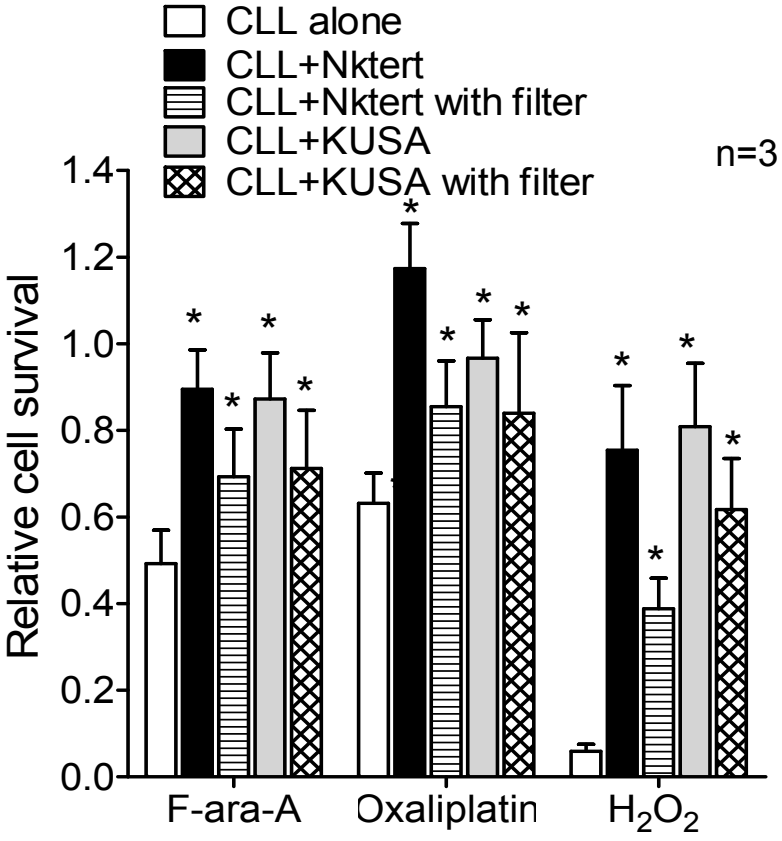

CLL alone
CLL+Nktert
CLL+Nktert with filter
CLL+KUSA
CLL+KUSA with filter
n=3
Relative cell survival
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
F-ara-A
Oxaliplatin
H2O2

Figure 23. Phenotype of CLL cells alone or co-cultured with bone marrow stromal cells (HS5, StromaNKtert, KUSA-H1). Figure displays phase-contrast photomicrophraphs that depict the morphologic appearance of CLL cells cultured alone or co-cultured with a layer of stromal cells. Cells were imaged in medium using a phase-contrast microscope with a 10X objective lens. Images were captured with a Nikon digital camera with the use of Camera Control Pro software (Nikon); when necessary, Adobe Photoshop 9.0 (Adobe Systems) was used for image processing.

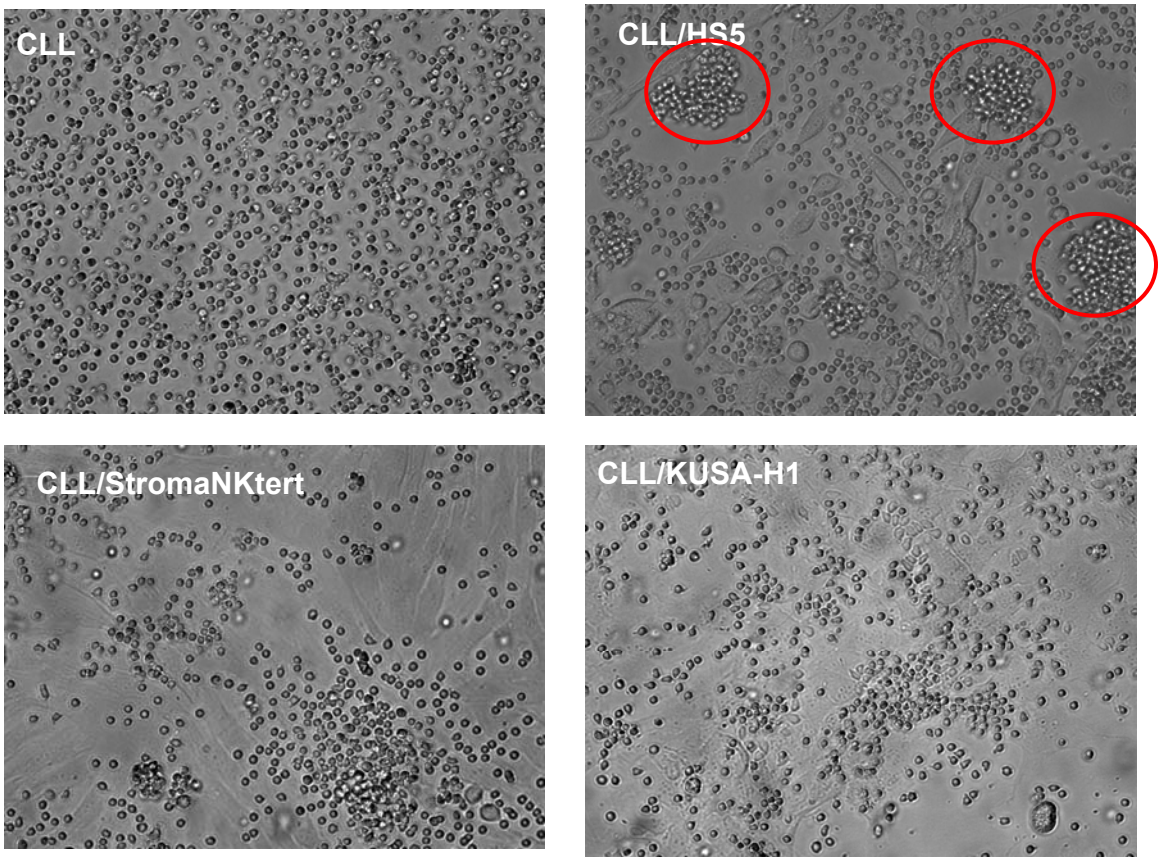
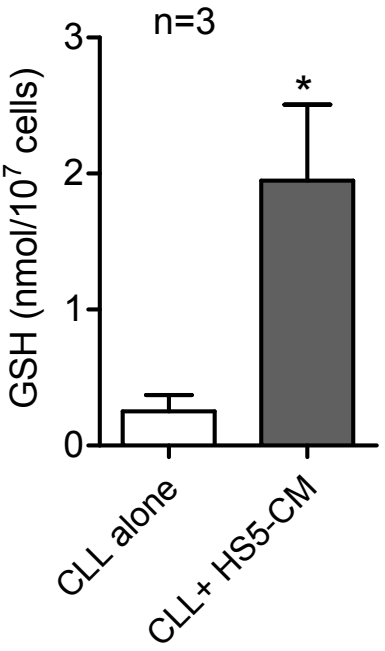
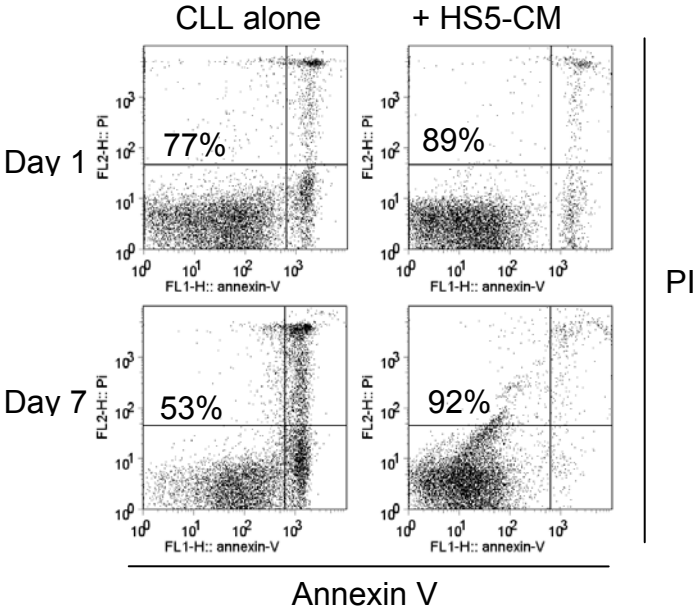

CLL
CLL/HS5
CLL/StromaNKtert
CLL/KUSA-H1

Figure 24. Soluble factor of HS5 stromal cells maintains GSH level of CLL cells and protects CLL cells from spontaneous apoptosis. (A) Comparison of GSH levels in CLL cells after cultured in regular medium or in HS5-conditioned medium (HS5-CM) for 72 h. *, $p<0.05$. **(B)** Annexin V-PI assay of CLL cell viability after culture in regular medium or in HS5-conditioned medium for 1 or 7 days. The number in each dot blot indicates % of viable cells (annexin-V/PI double negative).

A


n=3
GSH (nmol/10^7 cells)
3
2
1
0
*
CLL alone
CLL+ HS5-CM

B


CLL alone
+ HS5-CM
Day 1
Day 7
77%
89%
53%
92%
FL2-H:: PI
FL1-H:: annexin-V
PI
Annexin V

5. The low-molecular-weight fraction of the stromal medium enhances GSH synthesis in CLL cells and promotes cell survival.

I next investigated what was (were) the effective factor(s) in the conditioned medium of stromal cells. In light of the reports that cytokines and growth factors secreted by the accessory cells of tumor microenvironment protect leukemia cells for survival and confer drug resistance [14], firstly I measured the relative levels of the cytokines and chemokines secreted in the conditioned medium of single CLL cell cultures, single HS5 cell cultures and CLL/HS5 cell co-cultures respectively, by using the Human Cytokine Array Panel A (R&D Systems). Using this technology, the relative levels of up to 36 cytokines in a single sample can be profiled simultaneously (C5a, CD40 Ligand, G-CSF, GM-CSF, GROα, I-309, sICAM-1, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32α, IP-10, I-TAC, MCP-1, MIF, MIP-1α, MIP-1β, Serpin E1, RANTES, SDF-1, TNF-α, sTREM-1). Cultured alone, CLL cells secreted MIF (GIF, DER6) (**Fig 25B**), while HS5 cells secreted a variety of cytokines that are listed in **Fig 25**. Notably, no difference of cytokine secreting pattern was detected by this array in the medium of single HS5 cell cultures and CLL/HS5 cell co-cultures (**Fig 25C, D**). This observation indicated that no specific cytokines included in this array were secreted in response to CLL and HS5 cell interactions.

Because bone marrow stromal cells may release various factors including high molecular weight matrix factors, cytokines, growth factors, and low molecular weight amino acids and lipids [109, 117-120], to not miss the other possibilities, I separated the soluble factors in the HS5-conditioned medium into high-molecular-weight (HMW) and low-molecular-weight (LMW) fractions using the Amicon centrifugal filter devices with a 3-

kDa molecular cutoff, and tested their effect on CLL survival in the presence of drug incubation. Surprisingly, cytotoxicity analysis showed that it was the LMW fraction (< 3 kD) that provided most of the protective effect against drug-induced apoptosis, whereas the HMW fraction showed little protective activity (**Fig 26**). Consistently, incubation of CLL cells with LMW fraction helped the maintenance of cellular GSH pool at a high level (2.2 nmole/10^7 cells), similar to the GSH content in CLL cells cultured with unfiltered HS5-conditioned medium (**Fig 27**). In contrast, CLL cells cultured in regular medium showed a severe loss of GSH during the 3-day incubation, and the HMW faction of HS5-conditioned medium provided minimum protection against GSH loss (**Fig 27**). These data together suggest that a LMW component(s) of less than 3 kDa might play a key role in mediating the protective effect.

Since GSH is a small peptide (MW = 0.307 kDa) that can readily pass through the filter, I tested if HS5 stromal cells might secrete GSH into the medium for uptake by CLL. First, I used a common assay (the "end-point" method) to measure GSH and other reactive thiols in the medium of CLL culture with or without HS5. The results showed that HS5 significantly increased the thiol contents in the culture medium as well as in the CLL cells (**Fig 28**). However, since the end-point method utilized the Ellman's reaction to detect total free thiols including GSH, it became important to test if the stromal-induced elevation of thiol signals was due to elevated GSH or an increase in other LMW thiols such as cyteine. Thus, I used the kinetic method to specifically quantify GSH signal. As shown in **Fig 29A** (top panel), the slop of the kinetic reaction driven by GSH in the cell extracts of CLL cells co-cultured with HS5 was significantly higher than that of CLL cells cultured alone, confirming the increase of cellular GSH under co-culture

conditions. **Fig 29B** shows the quantitative data of three independent measurements of GSH in CLL cell extracts. The presence of stromal cells enhanced GSH levels in CLL cells by 4-5 folds. The kinetic method did not detect a significant GSH signal in the CLL culture medium or in the co-culture medium, as evidenced by the flat curves (**Fig 29A**, lower panel). These data suggest that the increased thiol signal in the stromal medium detected by the end-point method was not GSH, and was due to the presence of other low molecular weight thiols.

Using various concentrations of standard GSH in the medium, I determined that the minimum detection limit of this GSH assay was 0.3 μM. To detect GSH in the stromal conditioned medium, I increased the density of stromal cells and reduced the culture medium volume so that the cell/medium ratio increased by 2-4 folds. Under these conditions, GSH in the stromal conditioned medium could be detected (**Fig 30**). After normalization by the cell/medium ratio, the GSH levels in the stromal medium of normal cell density were 0.22 μM, 0.29 μM, and 0.32 μM for HS5, NKtert, and KUSA-H1 cells, respectively. Interestingly, such low concentrations of GSH were not sufficient to promote CLL cell viability, as evidenced by the failure of 2 μM GSH to enhance CLL cell survival in the absence of stromal cells (**Fig 31A**). Interestingly, low concentrations of GSH (2-10 μM) in the medium did not promote GSH synthesis in CLL cells, while higher concentrations (100-2000 μM) of exogenous GSH substantially enhanced the cellular GSH contents in CLL cells (**Fig 31B**). This was consistent with the observation that high concentration of exogenous GSH in the medium could promote CLL survival (**Fig 18**).

The above observations suggest that direct release of GSH into the medium by HS5 cells was unlikely the major mechanism by which the stromal cells enhanced GSH in CLL cells. I then tested if the stromal cells could indirectly promote GSH synthesis in CLL cells. GSH is synthesized by two sequential reactions catalyzed by γ-glutamylcysteine liganse (GCL) and glutathione synthetase (GS). The rate-limiting enzyme GCL is a heterodimer consisting of GCLC (catalytic) and GCLM (modulating) subunits, with the catalytic subunit GCLC being the rate-limiting component. Western blot analysis showed that the expression of GCLC was readily detectable in CLL cells, and that the presence of bone marrow stromal cells did not increase the expression of GCLC in 16 different CLL patient samples (**Fig 32**), suggesting that this rate-limiting enzyme was unlikely to be involved in stromal promotion of GSH synthesis in CLL cells.

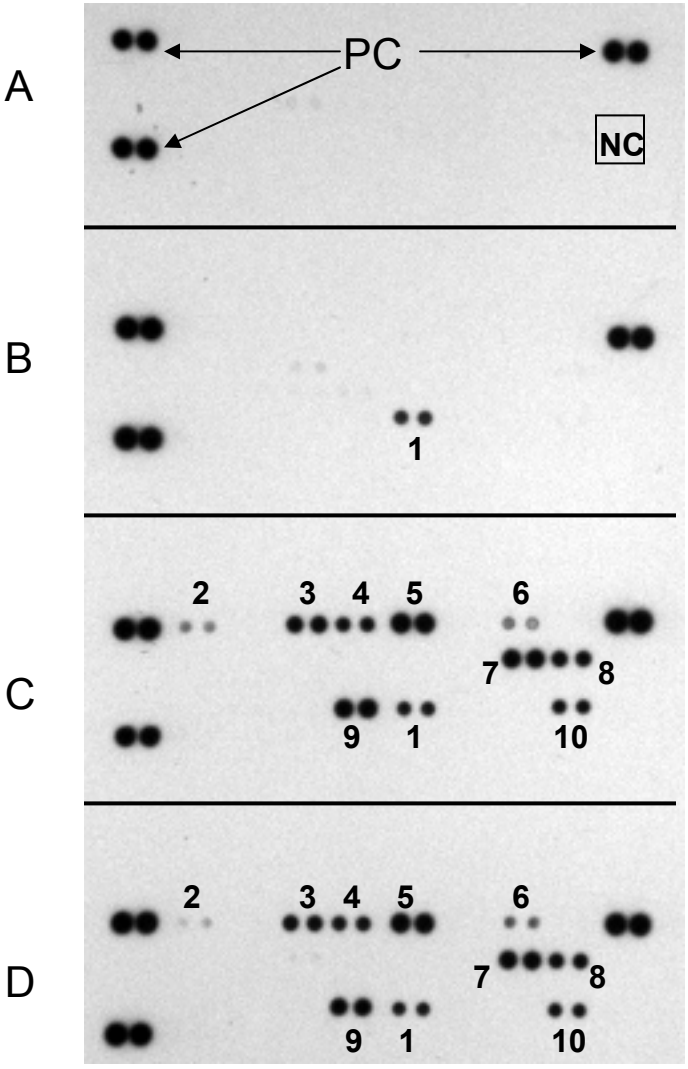
Figure 25. Cytokine array of conditioned medium from CLL cells, HS5 stromal cells, and CLL cells co-cultured with HS5 stromal cells. Secreted cytokines were detected using Human Cytokine Array Panel A from R&D systems (Minneapolis, MN). CLL cells and HS5 stromal cells were cultured alone or co-cultured for 3 days. Cell culture supernatants were collected and particulates were removed by centrifugation and assayed immediately according to the procedures recommended by the manufacture. Medium alone was examined as control.

A: Medium alone, RPMI 1640, 10% FBS.

B: Conditioned medium from CLL cell cultures on day3, cell density was $1X10^6$ cells/ml.

C: Conditioned medium from HS5 cell cultures on day3, cell density was $1.8X10^5$ cells/ml.

D: Conditioned medium from CLL and HS5 cell co-culture on day3.



PC	Positive Control
1	MIF (GIF,DER6)
2	C5a
3	G-CSF
4	GM-CSF
5	GROa (CXCL1)
6	sICAM1 (CD54)
7	IL6
8	IL8
9	MCP1 (CCL2)
10	Serpin E1 (PAI1)
NC	Negative Control

Figure 26. The low-molecular-weight fraction of stromal-conditioned medium promotes CLL cells survival. HS5 stromal cells were cultured until 80% confluence and switched to serum-free medium for 3 days. The conditioned medium was harvested, cleared by centrifugation, and then loaded to the reservoir chamber of the Amicon Ultra-15 centrifugal filter unit with molecular weight cut-off of 3-kDa (Millipore corporation, Billerica, MA). The tube was centrifuged at 4000x g at 4°C for 15min. The concentrated liquid remaining in the upper reservoir (high-molecular-weight fraction) was collected and reconstituted with serum-free medium to same volume before centrifugation. The components that passed the filter were collected as low-molecular-weight fraction. Both fractions were used to culture CLL cells immediately to test their effect on the viability CLL cells exposed to oxaliplatin (20 μM, 48 h). Cell viability was measured by flow cytomotry with annexin-V/PI staining.

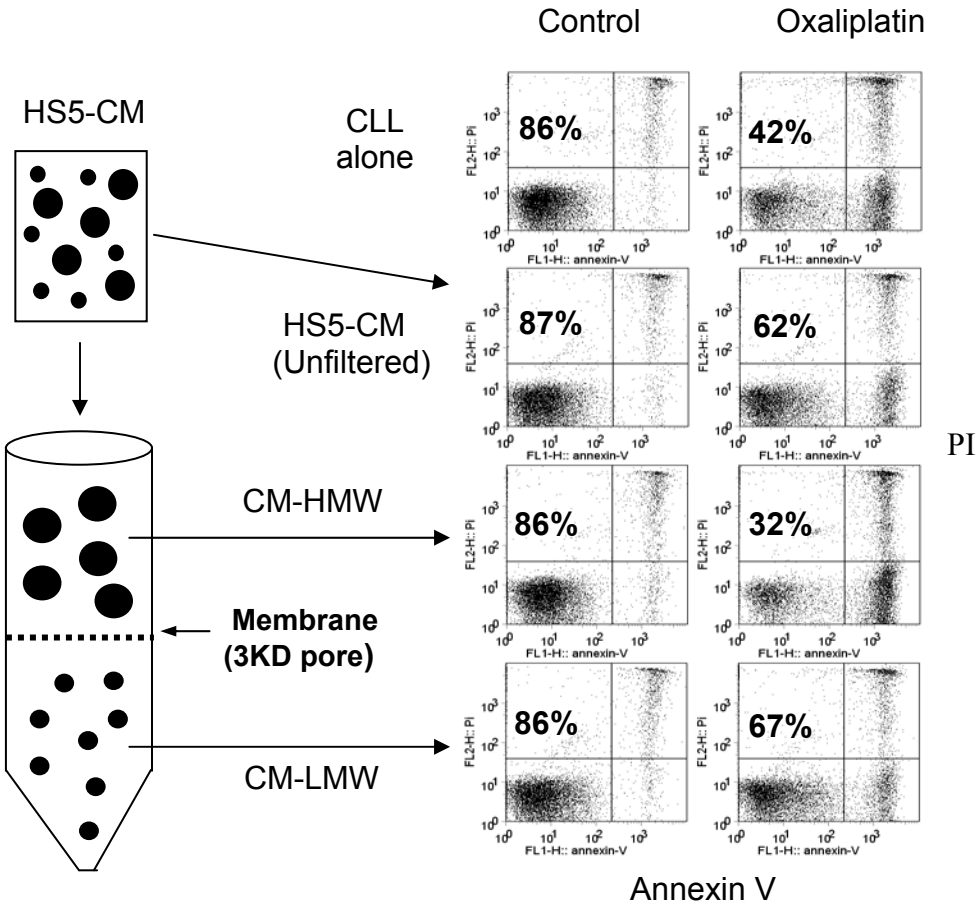

Control
Oxaliplatin
HS5-CM
CLL alone
HS5-CM (Unfiltered)
CM-HMW
Membrane (3KD pore)
CM-LMW
86%
42%
87%
62%
86%
32%
86%
67%
FL2-H:: PI
FL1-H:: annexin-V
PI
Annexin V

Figure 27. The low-molecular-weight fraction of stromal-conditioned medium enhances GSH synthesis in CLL cells. Serum-free HS5 cell conditioned medium was prepared sorted intro high-molecular-weight fraction and low-molecular-weight fraction by the same method described in Figure 23. Both fractions were used to culture CLL cells immediately. GSH levels in CLL cells cultured in different fractions of stromal-conditioned medium were examined on day 3. Each bar shows mean ±SEM of 3 experiments using 3 different CLL samples.

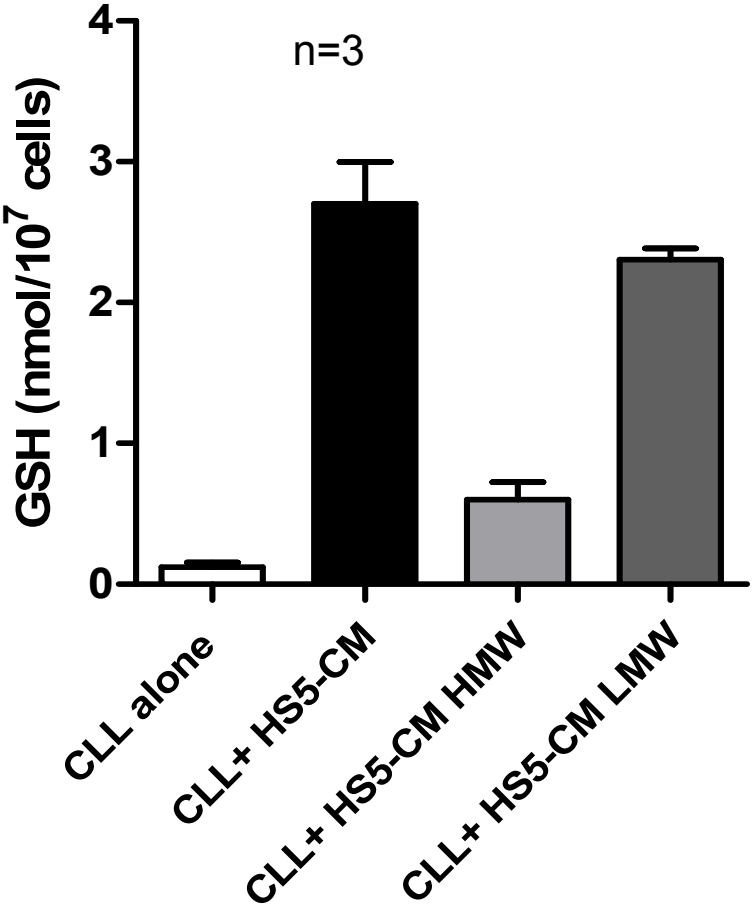

n=3
GSH (nmol/10^7 cells)
4
3
2
1
0
CLL alone
CLL+ HS5-CM
CLL+ HS5-CM HMW
CLL+ HS5-CM LMW

Figure 28. Determination of thiol by end-point method. Comparison of thiol levels in CLL cells or in the medium cultured with or without HS5 stromal cells for 72 h. The end-point method was used to measure thiol levels as described in Methods. Bar graphs of mean ±SEM from 3 experiments with 3 different CLL samples are shown (**, $p < 0.01$; ***, $p < 0.001$).

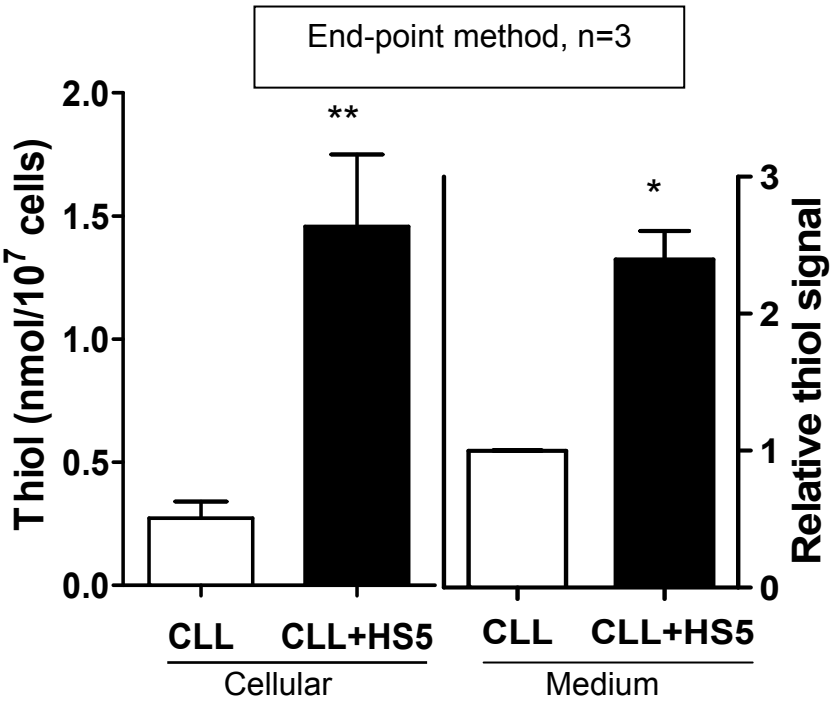
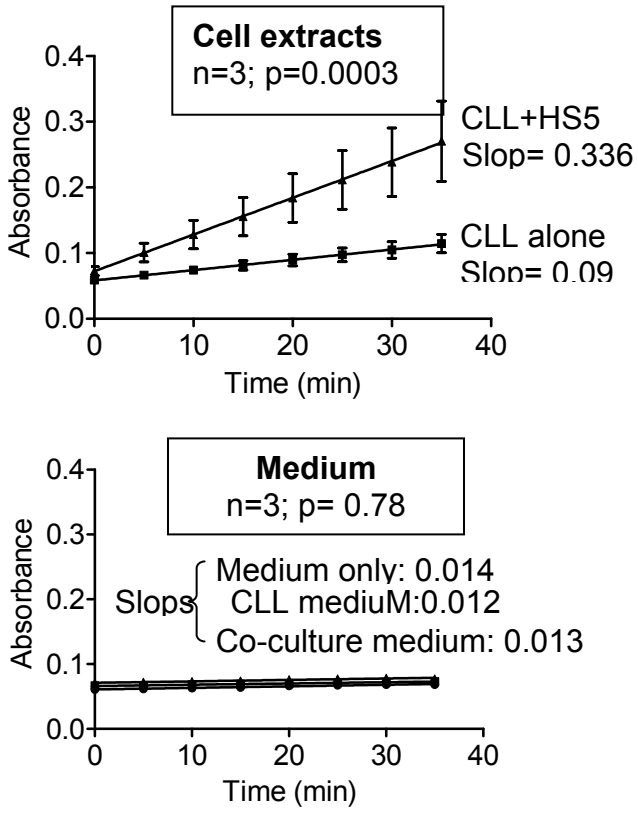

End-point method, n=3
Thiol (nmol/10^7 cells)
Relative thiol signal
2.0
1.5
1.0
0.5
0.0
3
2
1
0
**
*
CLL
CLL+HS5
CLL
CLL+HS5
Cellular
Medium

Figure 29. Determination of thiol by Kinetic method. (A) Kinetic measurement of GSH contents in CLL cells (upper panel) and in culture medium (lower panel) under the same culture conditions as in Fig 24. The slops reflect the kinetic reactions driven by GSH. **(B)** Quantitative comparison of GSH levels in CLL cells and in medium cultured with or without HS5 cells for 72 h. GSH concentration was determined by kinetic method. Each bar shows the mean ±SEM of 3 experiments using different CLL samples (**, $p < 0.01$; O, undetectable).

A


Cell extracts
n=3; p=0.0003
CLL+HS5
Slop= 0.336
CLL alone
Slop= 0.09
Absorbance
Time (min)
Medium
n=3; p= 0.78
Slops
Medium only: 0.014
CLL mediuM:0.012
Co-culture medium: 0.013
Absorbance
Time (min)

B

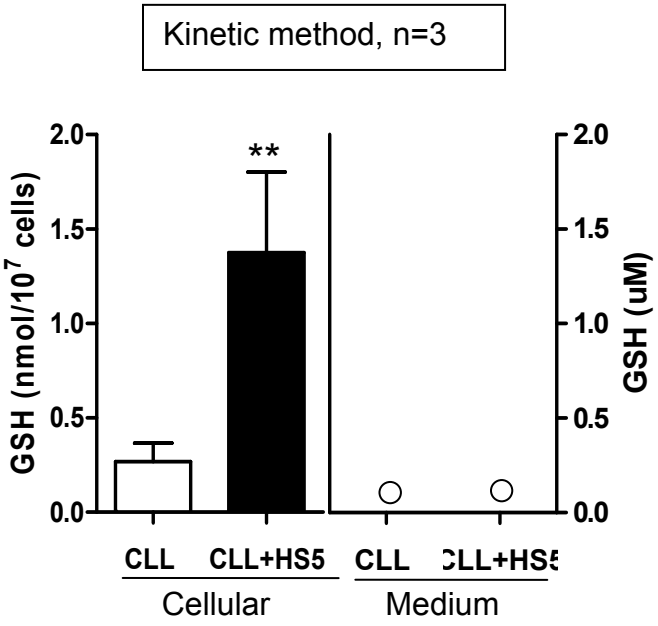
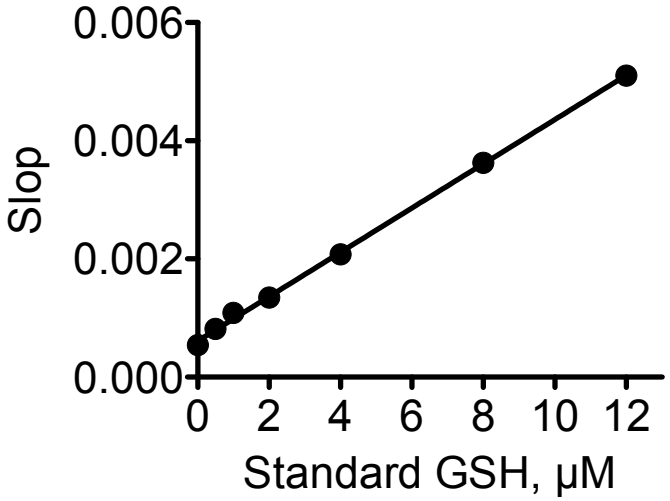
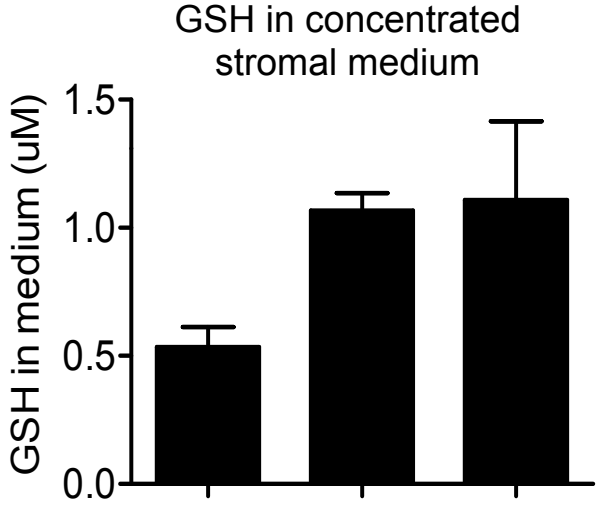

Kinetic method, n=3
**
GSH (nmol/10^7 cells)
GSH (uM)
CLL
CLL+HS5
CLL
CLL+HS5
Cellular
Medium

Figure 30. GSH contents in the conditioned medium of bone marrow stromal cells. GSH levels in the stromal conditioned medium from HS5, Nktert, and KUSA-H1 stromal cells. The panel on the left shows the standard curve of this assay. The conditioned medium was obtained under high cell density culture conditions as described in the text, and the normalized GSH levels in the culture medium under normal cell density were calculated using the corresponding cell/medium ratio for each cell line.

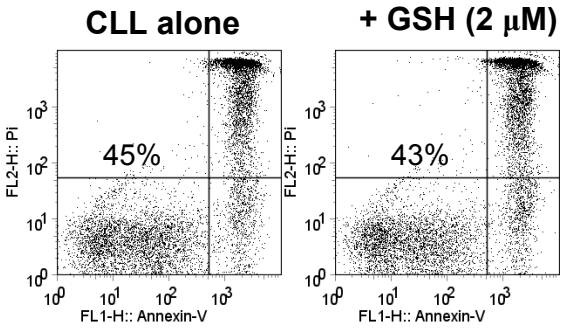




Stromal cells:	HS5	NKtert	KUSA
Dilution factor:	2.3	3.7	3.5
Normalized GSH:	0.22	0.29	0.32 (μM)

Figure 31. The effect of exogenous GSH on CLL cell viability. (**A**) Exogenous GSH at a low concentration (2 μM) did not promote CLL cell viability in culture without stromal cells. CLL cells were incubated with 2 μM GSH. Cell viability was assessed by annexin-V/PI staining. The number in each dot blot indicates % of viable cells (annexin-V/PI double negative). (**B)** Effect of various concentrations of GSH in the culture medium on the cellular GSH contents in CLL cells. CLL cells were incubated with 2 μM, 10 μM, 50 μM, 100 μM, 500 μM, 1mM, 2mM GSH. Cellular GSH levels in CLL cells were measured on day 3. (n=3 patient sample)

A


CLL alone
+ GSH (2 μM)
45%
43%
FL2-H:: PI
FL1-H:: Annexin-V

B

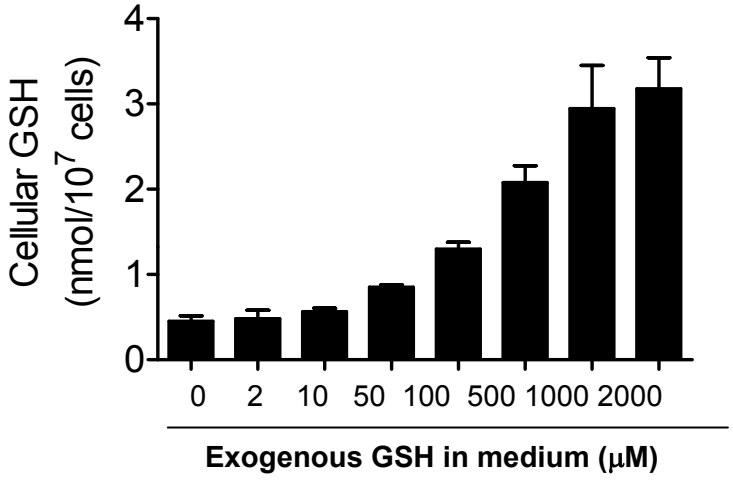
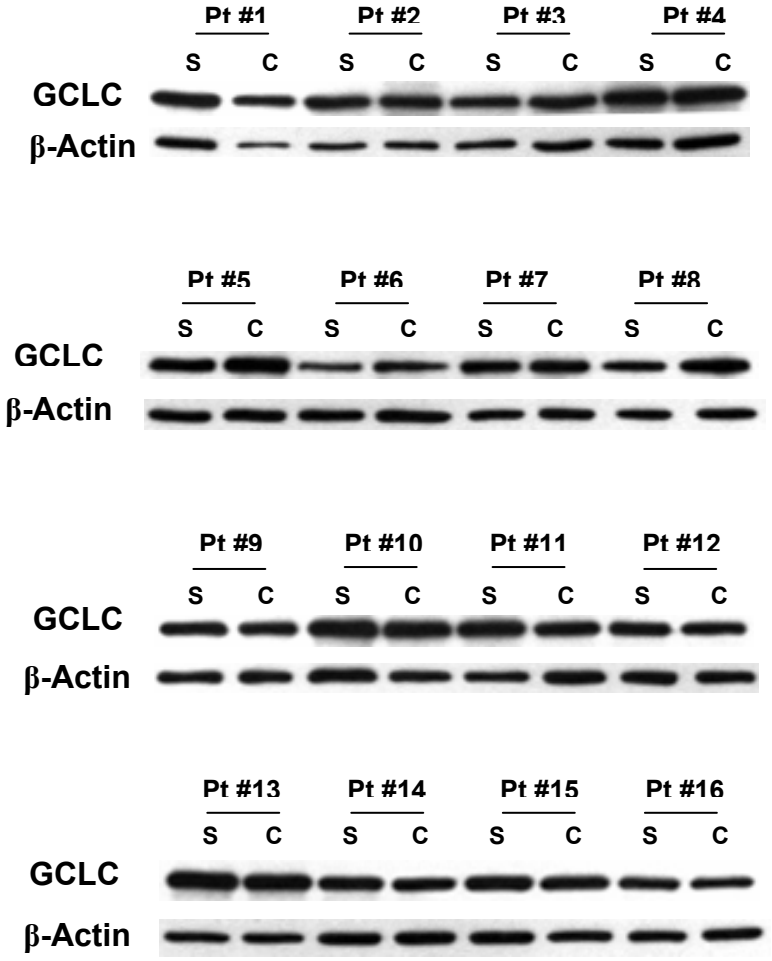

Cellular GSH
(nmol/10^7 cells)
0
1
2
3
4
0 2 10 50 100 500 1000 2000
Exogenous GSH in medium (μM)

Figure 32. Comparison of GCLC expression in CLL cells with or without bone marrow stromal cells. CLL cells isolated from peripheral blood of CLL patients were cultured alone or co-cultured with a layer of HS5 stromal cells for 3 days. CLL cells were removed from the stromal cells layer by gently shaking the flask and collecting CLL cells in the supernatant. Expression of GCLC in CLL cells from 16 patient samples in single culture (S, CLL alone) or in co-culture (C) with HS5 stromal cells were detected by western blot. Cell lysates were prepared and equal amounts of protein were electrophoresed on SDS-PAGE gels using standard conditions. The proteins were transferred to nitrocellulose membranes, which were probed with the following antibodies: GCLC; actin. Protein bands were visualized by chemiluminescent detection.


Pt #1
Pt #2
Pt #3
Pt #4
S C S C S C S C
GCLC
β-Actin
Pt #5
Pt #6
Pt #7
Pt #8
S C S C S C S C
GCLC
β-Actin
Pt #9
Pt #10
Pt #11
Pt #12
S C S C S C S C
GCLC
β-Actin
Pt #13
Pt #14
Pt #15
Pt #16
S C S C S C S C
GCLC
β-Actin

6. Generation of cysteine in the microenvironment by bone marrow stromal cells is critical to enhance GSH synthesis in CLL cells and promote their survival.

Among the three precursor amino acids (glutamate, cysteine, glycine) for GSH synthesis, the thiol-containing cysteine is chemically unstable and exists at a lower concentration than glutamate and glycine [116], and thus is a rate-limiting substrate for GSH synthesis. Because the HS5 stromal medium contained no detectable GSH (**Fig 29A-B**), I speculated that the LMW component in the stromal medium that enhanced GSH synthesis in CLL cells might be cysteine. To test this possibility, I first measured the LMW thiols in the acid-soluble extracts (to eliminate protein) of the culture medium from CLL or stromal cells (HS5, StromaNKtert, KUSA-H1), and showed that the medium contained only a trace amount of thiol before incubation with cells, and that there was a time-dependent increase in acid-soluble thiol concentrations in the stromal cell culture media (**Fig 33**), suggesting that all three bone marrow stromal cell lines were able to release LMW thiols, which were likely cysteine.

In order to detect cysteine in cell culture medium, two quick detection methods without derivatization were used. Firstly I performed the matrix-assisted laser desorption/ionization-time of flight mass spectrometer (MALDI-TOF MS) with the help of Dr. Hawke, the director of the proteomics facility in MD Anderson Cancer Center. Different matrices, such as dihydroxy- benzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (α-CHC) were tested after mixed with stromal cell conditioned medium with various ratios (1/10, 1/100, and 1/1000). It was found that the ratio of 1 to 100 was good for the crystallization of samples. However, the background noise of matrix in the low m/z area was too strong and covered the peak of cysteine. MALDI-TOF

mass spectrametry is good for analyzing protein which has high molecular weight that can avoid the background signal of matrix, but it is not a good method to detect small molecules like amino acid. Then I used electrospray ionization tandem mass spectrometry (ESI-MS/MS) to examine cysteine in culture medium by direct sample injection. Preliminary test by using pure cysteine showed that the transition pair of cysteine is 122/105, 76. By monitoring this transition pair, I found that the detection limit of pure cysteine prepared in water is 4 μM at the elution time of 3.5min (**Fig 34A**). However, when stromal cell conditioned medium was injected, no absorbance was detected around 3.5min (**Fig 34B**). Interestingly, cysteine signal was undetectable either when pure cysteine was prepared in regular culture medium. Since cell culture medium contains high concentration of salt, it is very likely that the salt in medium influences the ionization process and detection.

I then collected the stromal cell culture medium for metabolic profile analysis by NMR (Chenomx Inc. Edmonton, Canada), and showed that cystine was decreased in the stromal culture medium, indicating its utilization by the bone marrow stromal cells (**Fig 35A**). No cysteine signal was detectable by NMR analysis under this assay conditions (minimum detection limit was 2 μM) due to the instability of this compound in the medium during shipment, storage, and the required de-protein processing before NMR analysis (**Fig 35B**).

Luckily, MD Anderson bought a new LC-MS/MS system with a bigger C18 column with enhanced retention capacity. Based on the previous experience, the working model was proven finally. Stromal cell-conditioned medium was collected, cleared by centrifugation, and diluted 10:1 in buffer prior injection. By comparing the full scan of

MSMS of 122 ion (cysteine ion), the fingerprint of the daughter ions of the stromal cell-conditioned medium was exact the same with that of the authentic cysteine (**Fig 36A**), indicating that cysteine was detected in stromal cell-conditioned medium. This ionization pattern was not observed in control medium. The equation from the standard curve from 0.1 μM to 10 μM was used to calculate the concentrations of cysteine in the samples (**Fig 36B**). These samples were analyzed in triplicate. The result showed that cysteine concentration in control medium was low (0.25 μM), however, cysteine appeared in stromal cell-conditioned medium at a concentrations of 10-40 μM (**Fig. 36C**), which indicating that the protein-free thiol secreted by stromal cells was cysteine.

To further investigate the role of stromal-secreted cysteine in CLL survival and GSH synthesis, I tested the effect of exogenous cysteine in primary CLL cell culture without bone marrow stromal cells. Due to its instability, cysteine was added to the culture medium every 24 h for 3 days. Exogenous cysteine (50 μM) substantially increased the GSH content in CLL cells to a level comparable to that observed in CLL cells co-cultured with stromal cells (HS5), and 200 μM cysteine led to a significantly higher GSH (**Fig 37**). The exogenous cysteine promoted resistance to apoptosis induced by F-ara-A and oxaliplatin (**Fig 38**). Since the human plasma contains 10-20 μM cysteine[121, 122] which reflects the steady-state levels of cysteine constantly produced by the stromal cells and oxidized extracellularly, I tested the long-term effect of such physiological concentrations of cysteine on CLL viability. As shown in **Fig 39**, daily addition of 10 μM of cysteine was able to enhance CLL viability (from 4% to 34%) without drug treatment but did not prevent drug-induced cell death. Higher concentrations (20-200 μM) of cysteine

exhibited further protection of CLL cell viability and also promoted drug resistance in a concentration-dependent manner.

In contrast, the presence of 200 μM cystine in the regular RPMI medium failed to maintain GSH level in CLL cells without HS5 (**Fig 37**, CLL alone), suggesting that only cysteine could be utilized by CLL cells. Chemical conversion of cystine to cysteine by adding the strong reducing agent 2-mercaptoethanol (2-ME, 20 μM) to the culture medium (**Fig 40A**) effectively increased GSH content in CLL cells (a 10-fold increase) without stromal cells (**Fig 40B**), further confirming the important role of cysteine. In cell viability study, CLL cells lost their viability in regular culture medium without 2-ME, and addition of 20 μM 2-ME conferred a striking protective effect that kept the majority of CLL cells viable for 3 weeks (**Fig 41A**). The importance of such chemical conversion was further demonstrated in a separate experiment, where 2-ME failed to protect CLL cells in the cystine-free medium (**Fig 41B**), indicating that cystine was required to be reduced to cysteine by 2-ME to provide the protective effect. The protective effect of 2-ME on B cell survival has been known for a long time, but the mechanism is still unclear. In this study, I found that the chemical conversion from cystine to cysteine by 2-ME is critical for 2-ME to protect B cells. Consistently, exogenous 2-ME provided significant protection against drug-induced apoptosis in CLL cells exposed to F-ara-A or oxaliplatin in regular culture medium containing 200 μM cystine (**Fig 42**).

Figure 33. Release of acid-soluble thiols into extracellular environment by 3 lines of bone marrow stromal cells (HS5, StromaNKtert, and KUSA-H1). The stromal cultures were replenished with fresh medium, and at the indicated time points aliquots of medium were removed, clarified by centrifugation, and extracted with sulfosalicylic acid to remove protein and neutralized with NaOH. Thiol contents were measured by reaction with freshly prepared DTNB by measuring absorbance at 412nm. Each sample was assayed in triplicate. The acid-soluble thiol concentration was calculated using cysteine as a reference standard. Medium alone without cells was incubated in parallel at the same time as a control. Each data point was the mean ±SEM of 3 separate experiments.

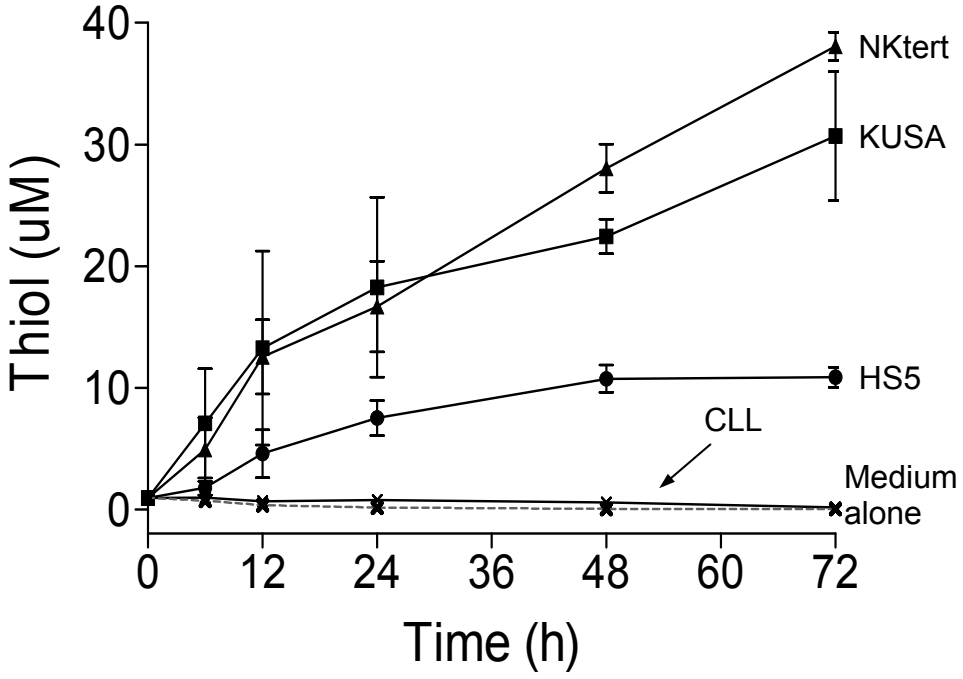
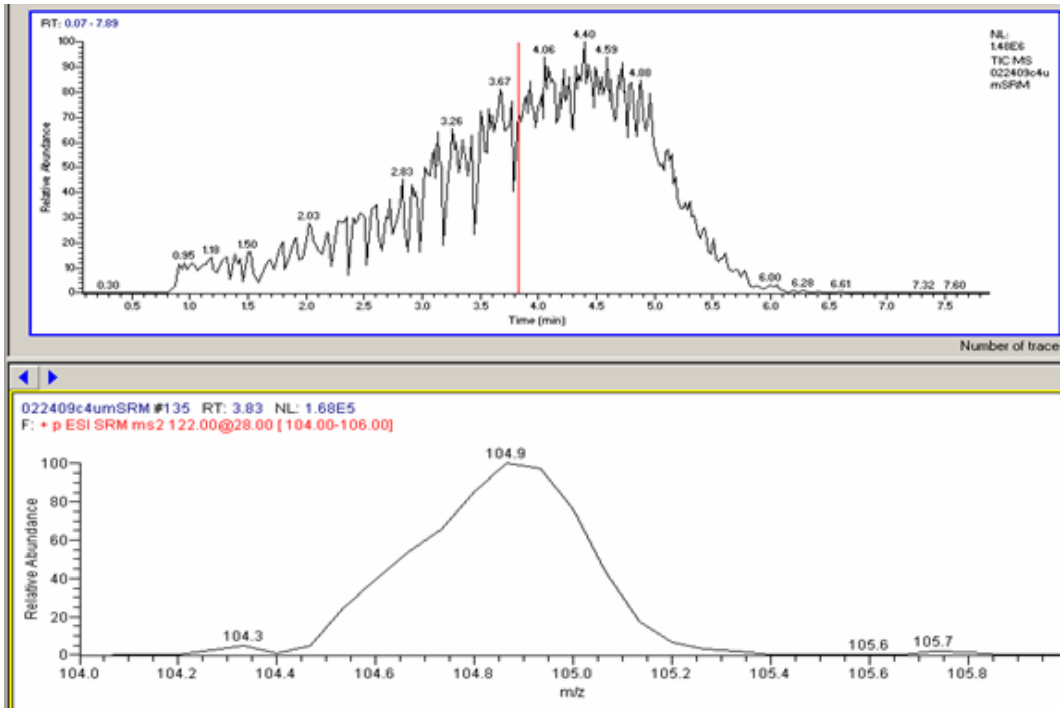

40
30
20
10
0
Thiol (uM)
0
12
24
36
48
60
72
Time (h)
NKtert
KUSA
HS5
CLL
Medium
alone

Figure 34. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis of standard cysteine and cysteine in HS5 stromal cell conditioned medium. Pure cysteine prepared in water (**A**) and stromal cell conditioned medium (**B**) were injected to mass spectrometry. Mass spectrometry was carried out in positive ion mode on a linear ion trap mass spectrometer, using a nanoelectrospray source for direct infusion of samples by static nanospray with isolation width as m/z 1.0, and acquisition time as 0-8 min. Electrospray voltage was 1 kV. Static nanoelectrospray needles were from Proxeon Biosciences.

A


RT: 0.07 - 7.89
Relative Abundance
Time (min)
NL:
1.48E6
TIC MS
022409c4u
mSRM
Number of traces
022409c4umSRM #135 RT: 3.83 NL: 1.68E5
F: + p ESI SRM ms2 122.00@28.00 [104.00-106.00]
104.9
104.3
105.6
105.7
m/z

B

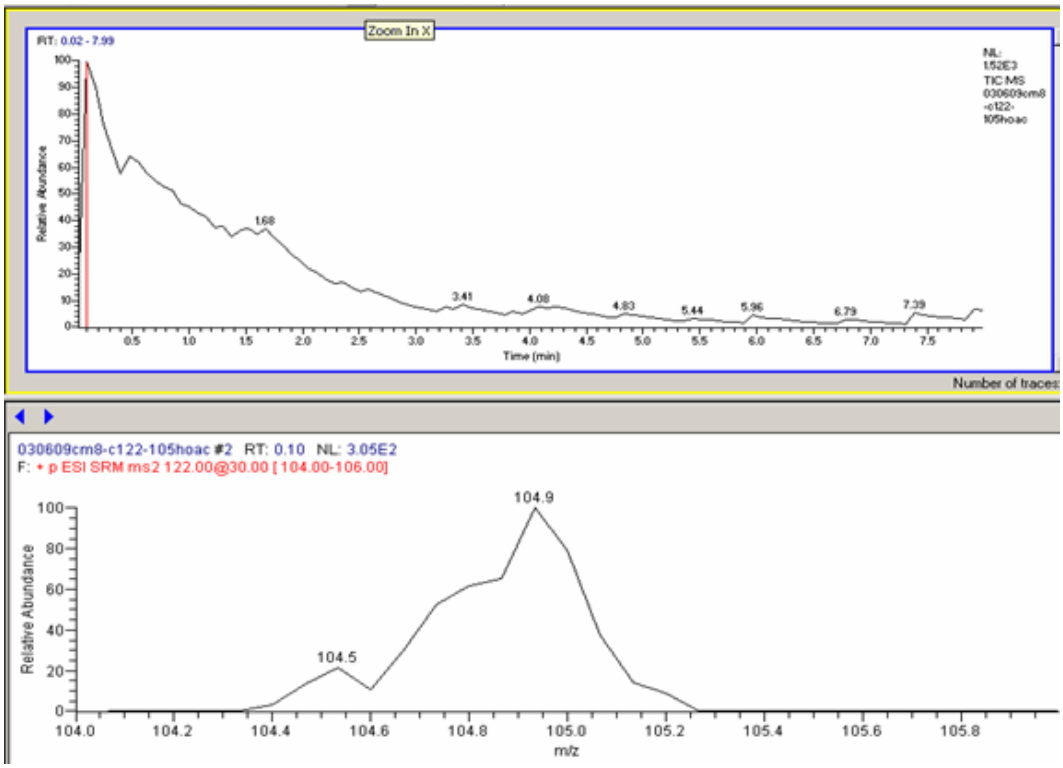
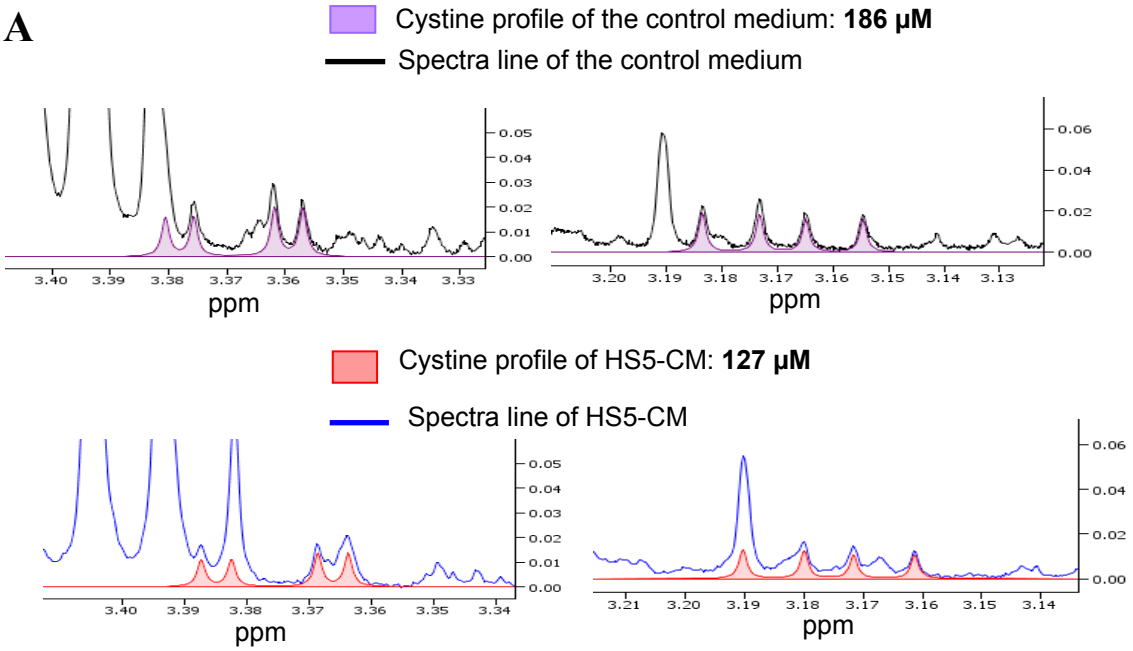

Zoom In X
RT: 0.02 - 7.99
Relative Abundance
Time (min)
NL:
1.52E3
TIC MS
030609cm8
-c122-
105hoac
Number of traces
030609cm8-c122-105hoac #2 RT: 0.10 NL: 3.05E2
F: + p ESI SRM ms2 122.00@30.00 [104.00-106.00]
104.9
104.5
m/z

Figure 35. NMR analysis of cystine and cysteine in HS5 stromal conditioned medium and control medium. The HS5 stromal conditioned medium (HS5-CM) and control medium (without cell culture) were collected and sent to Chenomx Inc (Edmonton, Canada) for NMR analysis. The media were deproteinated before analysis by 1H NMR spectroscopy. **(A)** Cystine signature profile and spectra line of the control medium (upper panel) and HS5-CM (lower panel). **(B)** Standard cysteine signature profile (green) and the spectra line of the control medium (black) and HS5-CM (blue). Segments of the spectra containing the clusters of cystine or cysteine are shown. There were no detectable spectra patterns of the control medium or stromal conditioned medium that match with the standard cysteine spectra profile, indicating a loss of cysteine to the level below the detection limit (2 mM) in the samples due to the instability of the compound.

A


Cystine profile of the control medium: 186 µM
Spectra line of the control medium
ppm
ppm
Cystine profile of HS5-CM: 127 µM
Spectra line of HS5-CM
ppm
ppm

B

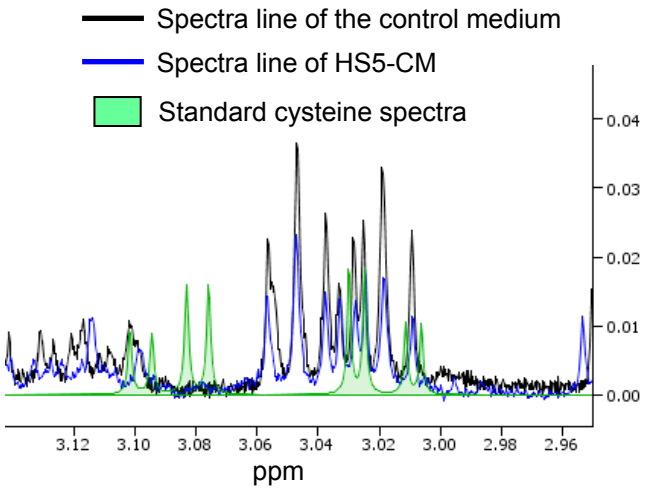
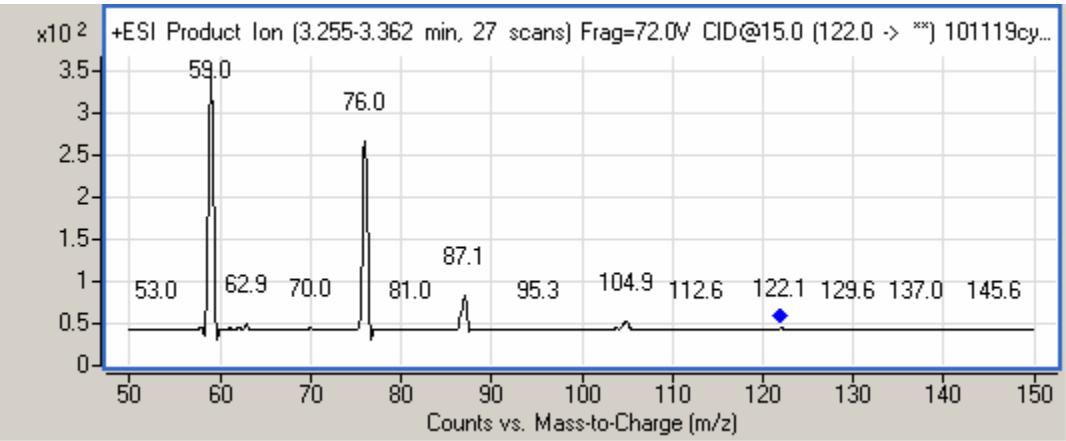

Spectra line of the control medium
Spectra line of HS5-CM
Standard cysteine spectra
ppm

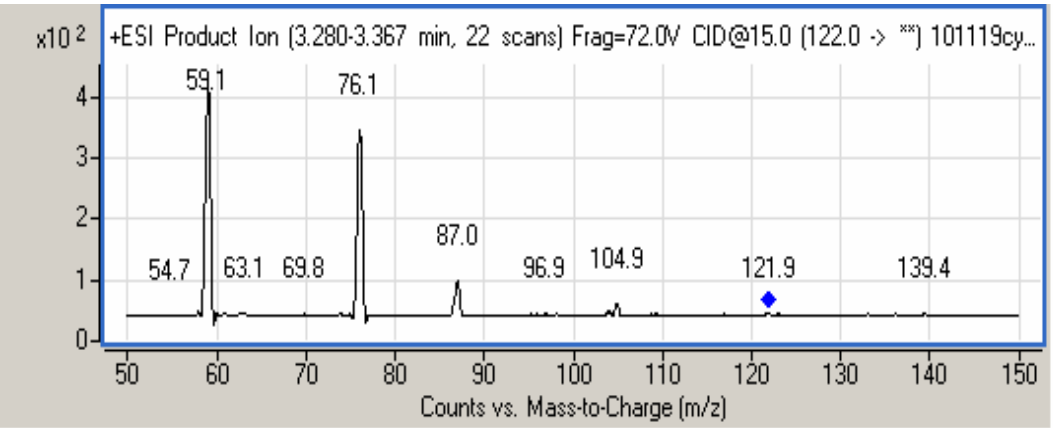
Figure 36. Analysis of Cysteine by LC-MS/MS on the Triple-Quadrupole Mass Spectrometer. (**A**) Full scan of MSMS of 122 ion of authentic cysteine and stromal cell-conditioned medium. Conditioned medium was diluted 10:1 in buffer prior injection. The singly-charged, protonated Cysteine ion (MH^+) was selected for fragmentation in an injection of the standard solution at 1μM concentration, and also for the sample injection. **(B)** Standard curve of cysteine. The equation from the standard curve from 0.1 μM to 10 μM was used to calculate the concentrations of cysteine in the samples. **(C)** Cysteine concentration in control medium and stromal cell-conditioned medium. These samples were analyzed in triplicate.

A

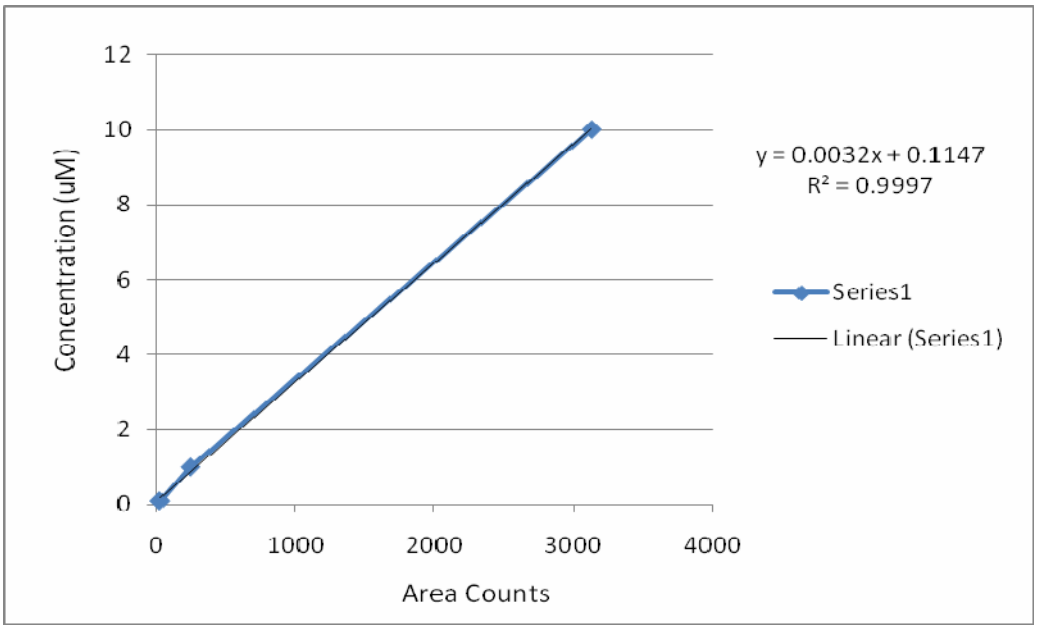
Authentic cysteine



Stromal cell-conditioned medium



B



C

	Control	HS5-CM	NKtert-CM
Cysteine (μM)	0.25	8.8	42.6
%sd	3.12	3.53	1.65

Figure 37. Extracellular cysteine enhanced CLL cellular GSH contents. CLL cells were isolated from peripheral blood of CLL patient and daily supplemented with 50-200 μM cysteine. CLL cells cultured alone or with a layer of HS5 stromal cells were used as controls for comparison. GSH assay was performed on day 3. Each bar represents mean ±SEM of 4 separate experiments (**, $p<0.01$; ***, $p<0.001$).

n=4

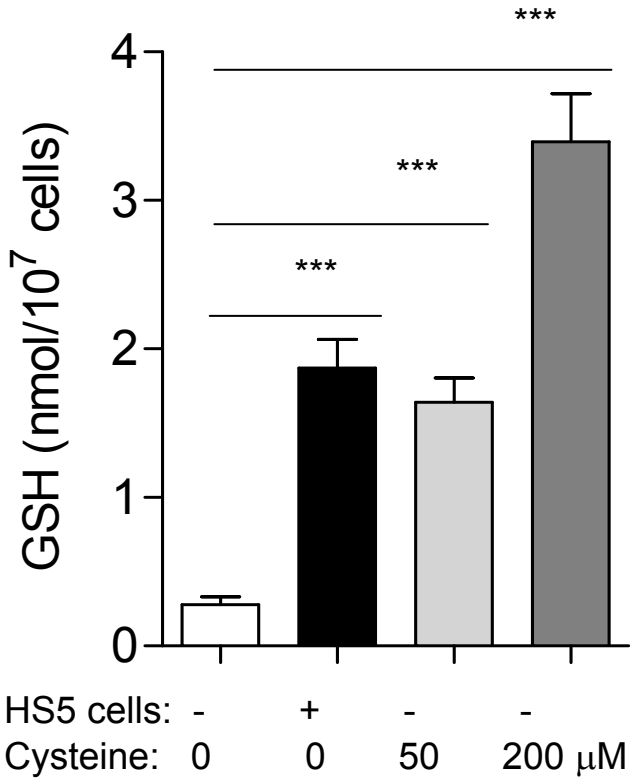
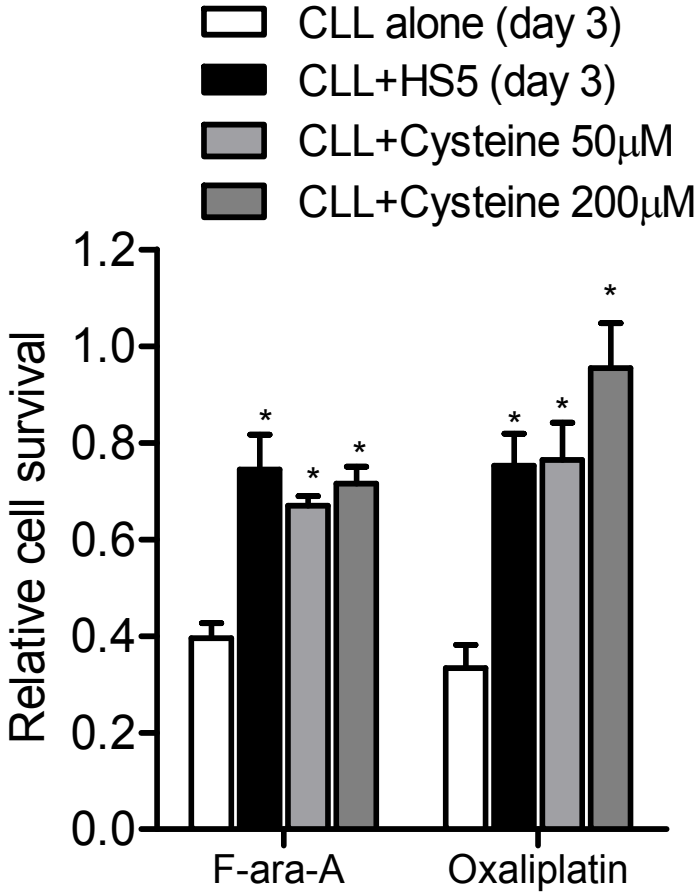


Figure 38. Extracellular cysteine conferred drug resistance to CLL cells. Cells were isolated from peripheral blood of CLL patient and cultured alone, with cysteine (50-200 μM, added daily), or with HS5 stromal cells for 24 h, then treated with 20 μM F-ara-A or 20 μM oxaliplatin for another 48 h, and analyzed for cell viability by flow cytometry after staining with annexin V and PI. The total incubation time was 3 days. Each bar represents mean ± SEM of 3 separate experiments (*, $p<0.05$).


CLL alone (day 3)
CLL+HS5 (day 3)
CLL+Cysteine 50μM
CLL+Cysteine 200μM
Relative cell survival
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
F-ara-A
Oxaliplatin

n=3

Figure 39. Effect of various concentrations of cysteine on CLL cell survival and drug sensitivity cultured without stromal cells. Fresh isolated CLL cells were incubated with 10, 20, 50, 100 μM cysteine. Cysteine was added daily continuously for 7 days and treated with 20 μM F-ara-A or 20 μM oxaliplatin for another 2 days. Cell viability was measured by flow cytometry analysis after staining with annexin V and PI on day 9. The representative dot plot was shown. The number in each dot blot indicates % of viable cells (annexin-V/PI double negative).

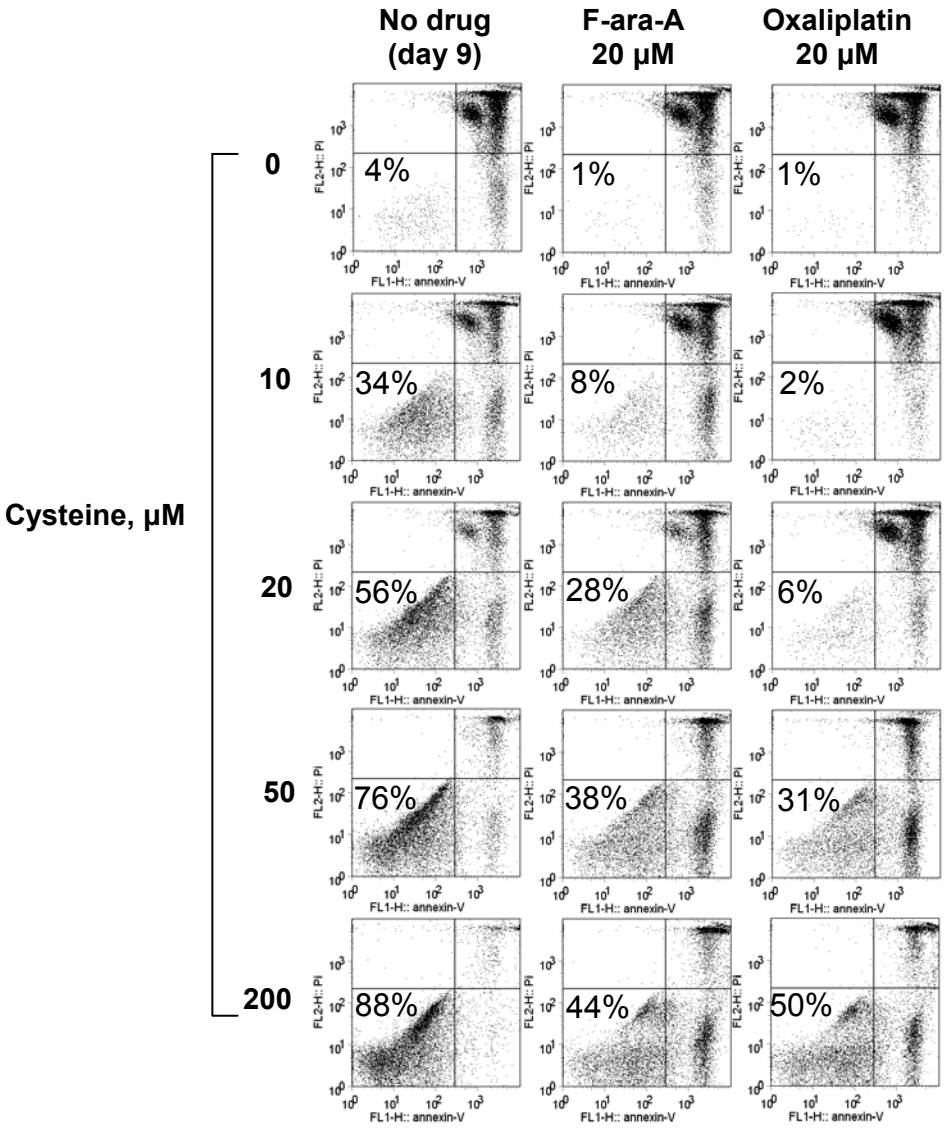
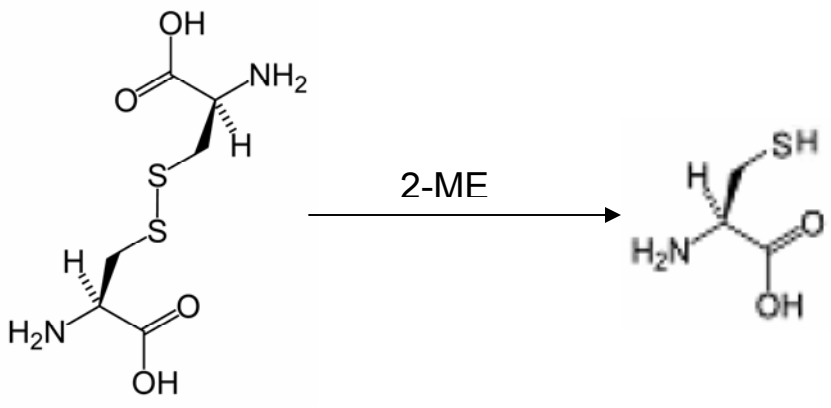

No drug
(day 9)
F-ara-A
20 μM
Oxaliplatin
20 μM
Cysteine, μM
0
10
20
50
200
4%
1%
1%
34%
8%
2%
56%
28%
6%
76%
38%
31%
88%
44%
50%
FL2-H:: PI
FL1-H:: annexin-V

Figure 40. Conversion of extracellular cystine to cysteine by 2-mercaptolethanol (2-ME) enhances GSH synthesis in CLL cells. (**A**) Chemical reduction of cystine to cysteine by 2-ME. (**B**) CLL cells were isolated from peripheral blood of CLL patient and cultured alone or with the supplementation of 20 μM 2-ME. Cellular GSH level was detected on day 3. (***, $p<0.001$, n= 3 different CLL samples).

A


OH
NH2
O
H
S
S
H
H2N
O
OH
2-ME
SH
H
H2N
O
OH

B

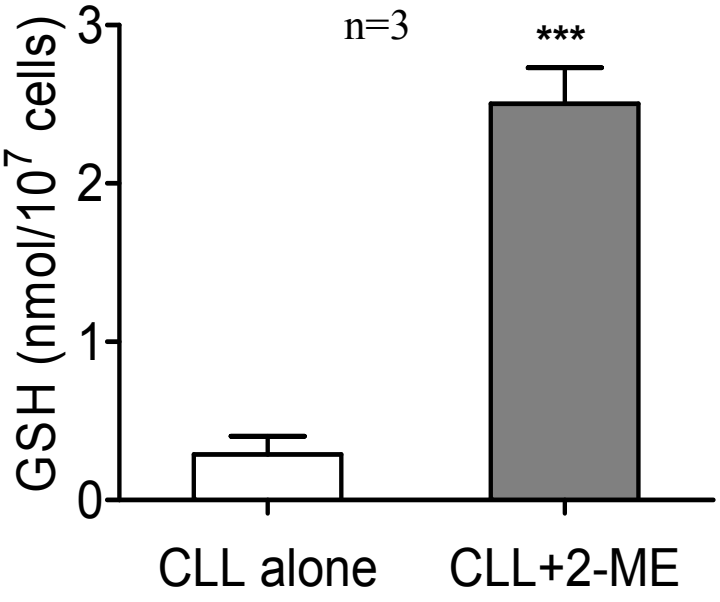
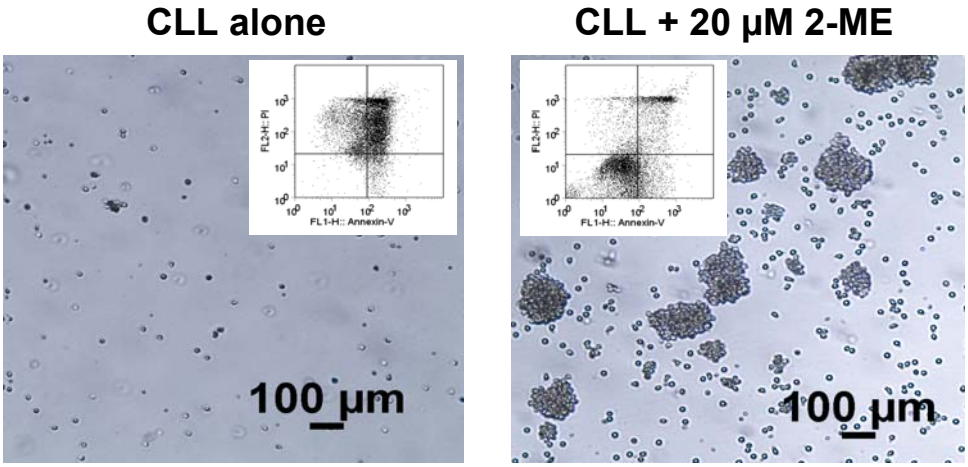

n=3
***
GSH (nmol/10^7 cells)
3
2
1
0
CLL alone
CLL+2-ME

Figure 41. 2-mercaptolethanol (2-ME) protects CLL cells for survival *in vitro* in the presence of cystine. (A) Conversion of cystine to cysteine in the culture medium by 2-ME (20 μM) promoted CLL cell long-term survival in culture with regular RPMI medium (containing 200 μM cystine). Cell viability was measured by flow cytometry analysis after staining with annexin V and PI. CLL cells were imaged in medium using a phase-contrast microscope with a 10X objective lens. Images were captured with a Nikon digital camera with the use of Camera Control Pro software (Nikon); when necessary, Adobe Photoshop 9.0 (Adobe Systems) was used for image processing. Flow cytometry and photographs were performed on day 20. **(B)** 2-ME failed to protect CLL cells in cystine-free medium.

A


CLL alone
CLL + 20 µM 2-ME
100 µm
100 µm

B

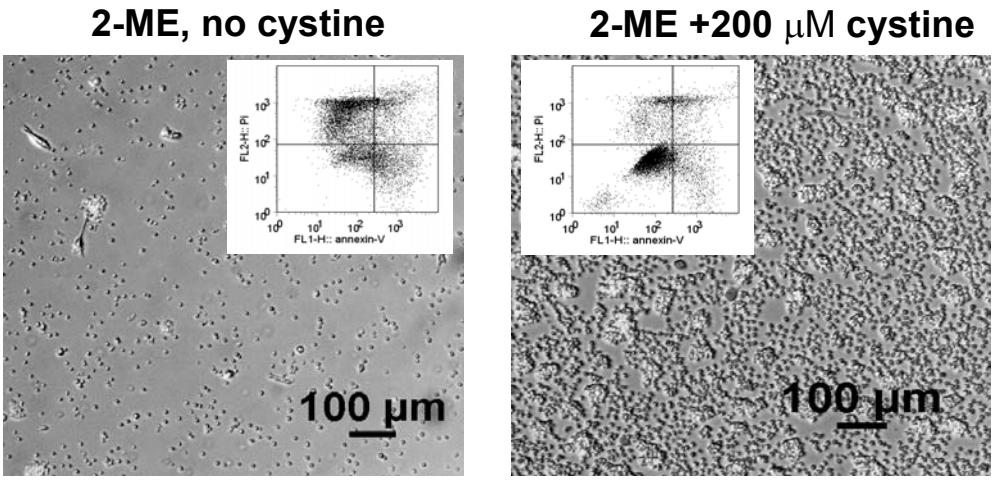
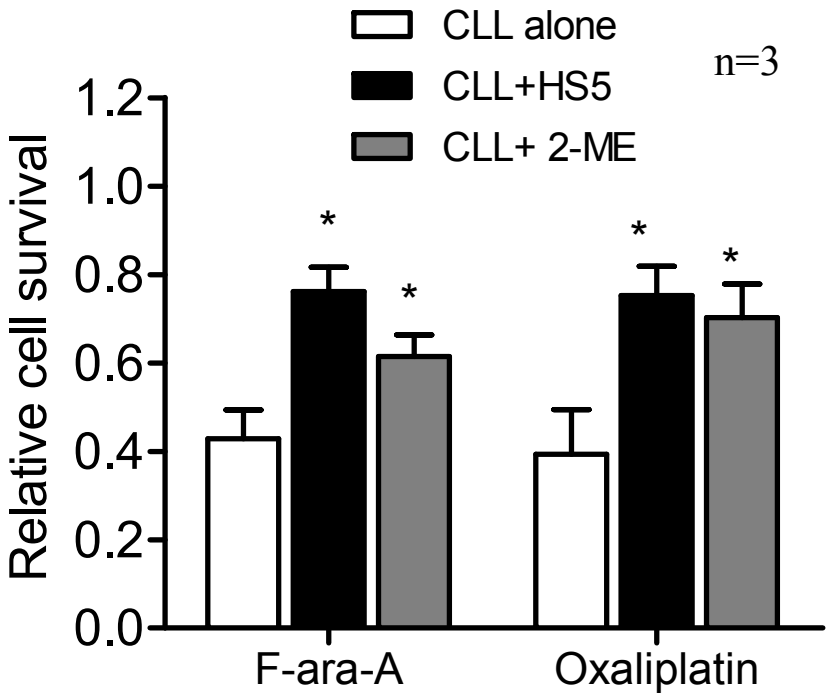

2-ME, no cystine
2-ME +200 µM cystine
100 µm
100 µm

Figure 42. Conversion of cystine to cysteine by 2-mercaptolethanol in culture medium confers drug resistance to CLL cells. CLL cells were isolated from peripheral blood of CLL patient and cultured alone, with 2-mercaptolethanl (2-ME, 20 μM), or with HS5 stromal cells for 24 h, and then treated with F-ara-A (20 μM) or oxaliplatin (20 μM) for another 48 h. cell viability was analyzed by flow cytometry after double staining with annexin-V/PI. Each bar represents mean ± SEM of 3 separate experiments using 3 different CLL patient samples (*, $p<0.05$; **, $p<0.01$).


CLL alone
CLL+HS5
CLL+ 2-ME
n=3
Relative cell survival
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
*
F-ara-A
Oxaliplatin

7. Biochemical pathway between bone marrow stromal cells and CLL cells.

I then investigated why CLL cells were highly dependent on cysteine in the medium to maintain GSH synthesis and cell survival. It is known that most cells are incapable of synthesizing cysteine and they obtain the GSH precursor by the uptake of extracellular cysteine and cystine through specific transporters [79-81]. Because cysteine is unstable, the stable cystine is the dominant precursor in the culture medium and in plasma, and is transported into the cells by a transporter known as Xc-. Within the cells, cystine is reduced to cysteine for GSH synthesis. The facts that extracellular cystine did not enhance GSH level in CLL cells and failed to protect them in the absence of stromal cells or 2-ME led us to postulate that CLL cells might not be able to take up cystine. I first tested the ability of CLL cells to utilize extracellular cystine in comparison with bone marrow stromal cells. Western blot analysis showed that the expression of xCT, the active subunit of cystine transporter Xc- [84], was highly expressed in the HS5 stromal cells but was dramatically diminished in CLL cells (**Fig 43**). I also analyzed the xCT mRNA expression levels in CLL cells and normal lymphocytes using the National Center for Biotechnology Information gene expression omnibus database (ID: GDS1454) that contained microarray data from 100 CLL patient samples and 11 healthy control samples [121], and found that CLL cells expressed a significantly lower xCT than normal lymphocytes in this data set ($p<0.001$).

Functional analyses were performed to further compare the ability of CLL cells and bone marrow stromal cells to take up extracellular cystine, using radioactive [^{14}C]-cystine as the substrate for quantitative measurement. As shown in **Fig 44**, CLL cells exhibited little uptake of [^{14}C]-cystine, whereas HS5 were highly effective in taking up [^{14}C]-

cystine, consistent with their high expression of Xc- transporter (**Fig 43**). Importantly, conversion [^{14}C]-cystine to [^{14}C]-cysteine by 2-mercaptolethanol significantly increased the uptake of the radioactive substrate (15-fold increase) by CLL cells in culture (**Fig 45**). Because HS5 cells expressed a high level of cystine transporter and exhibited efficient uptake of [^{14}C]-cystine whereas CLL cells could only import [^{14}C]-cysteine, I tested if the stromal cells could promote GSH synthesis in CLL cells by converting cystine to cysteine for CLL cells. CLL cells cultured alone or co-cultured with HS5 cells were incubated with the same amount of [^{14}C]-cystine, and the radioactive uptake in the CLL cells was determined after the leukemia cells in suspension were separated from HS5 stromal cells. As shown in **Fig 46**, there was a highly significant increase in the uptake the radioactive material by CLL cells in the presence of HS5, despite a consumption and retention of [^{14}C]-cystine by the stromal cells. Western blot analysis showed that co-culture with HS5 did not alter the expression of xCT transporter in CLL cells (**Fig 47**), excluding the possibility that the increased uptake of the radioactive material in CLL cells might be due to elevated expression of the transporter. To further confirm the important role of extracellular cystine in maintaining cell viability in the co-culture system, I incubated CLL cells with HS5 cells in a cystine-free medium, and showed that the absence of cystine in culture medium abolished the ability of HS5 cells to promote GSH synthesis in CLL cells (**Fig 48**), and abrogated the protective effect of HS5 cells on CLL survival in the presence of F-ara-A or oxaliplatin (**Fig 49**). Together these data indicate that CLL cells have low ability to take up cystine due to low expression of Xc- transporter, and that bone marrow stromal cells promoted GSH synthesis in CLL cells by taking up cystine and converting it to transportable cysteine for CLL cells.

However, when HS5 cells were cultured without cystine for 3 days, they lost viability and became detached from the culture dish (**Fig 50**), suggesting that cystine is an essential thiol source to maintain stromal cell survival. Similarly, when other two stromal cell lines (StromaNKtert and KUSA-H1) were cultured without cystine, they also lost they viability in 3 days (**Fig 51**). Addition of 5 μM cystine was able to support the survival of the stromal cells and moderately promote CLL cell viability in co-culture. Higher concentrations of cystine (50-200 μM) significantly promoted the viability of both stromal and CLL cells, even in the presence of 20 μM of F-ara-A or oxaliplatin (**Fig 51**). Although the lost of stromal cell viability in the absence of cystine seemed to complicate the data interpretation, the ability of exogenous cysteine to protect CLL cells without stromal cells (**Fig 39**) strongly suggest that the protective effect was mediated by cysteine. Obviously, the viable stromal cells were needed to convert cystine to cysteine when the culture medium only contained cystine.

Figure 43. Expression of the cystine transporter xCT in HS5 stromal cells and primary CLL cells. Cell lysates of HS5 stromal cells and CLL cells (n=10) were prepared and equal amounts of protein were electrophoresed on SDS-PAGE gels using standard conditions. The proteins were transferred to nitrocellulose membranes, which were probed with the following antibodies: xCT and actin. Protein bands were visualized by chemiluminescent detection.

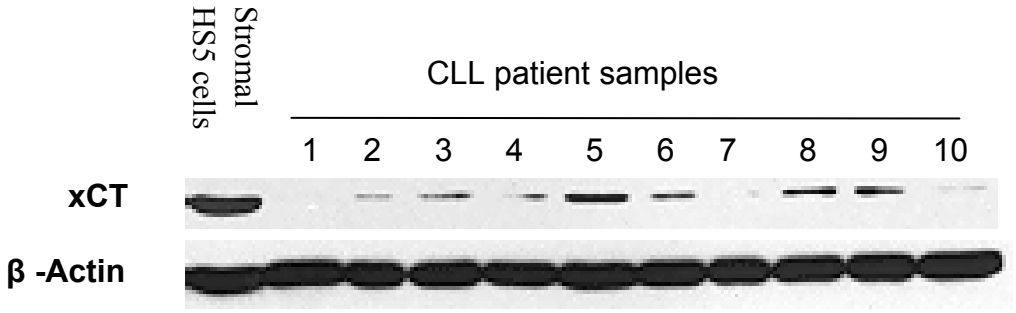

Stromal HS5 cells
CLL patient samples
1 2 3 4 5 6 7 8 9 10
xCT
β -Actin

Figure 44. Comparison of [14C] cystine uptake by HS5 stromal cells and CLL cells. Isolated CLL cells and HS5 stromal cells were incubated in fresh cystine-free RPMI 1640 supplemented with dialyzed 10% FBS, respectivley. [14C]-cystine (PerkinElmer, Waltham, MA) was at (0.2 μCi/ml) and incubated for 4 hours. The cells were washed twice with cold PBS. Cell pellets were resuspended in 200 μL PBS, lysed with 3 mL scintillation fluid, and radioactivity was measured by a Beckman liquid scintillation counter. Bar graph of mean ±SEM of 3 separate experiments using CLL cells from 3 different patients is shown (***, $p<0.001$).

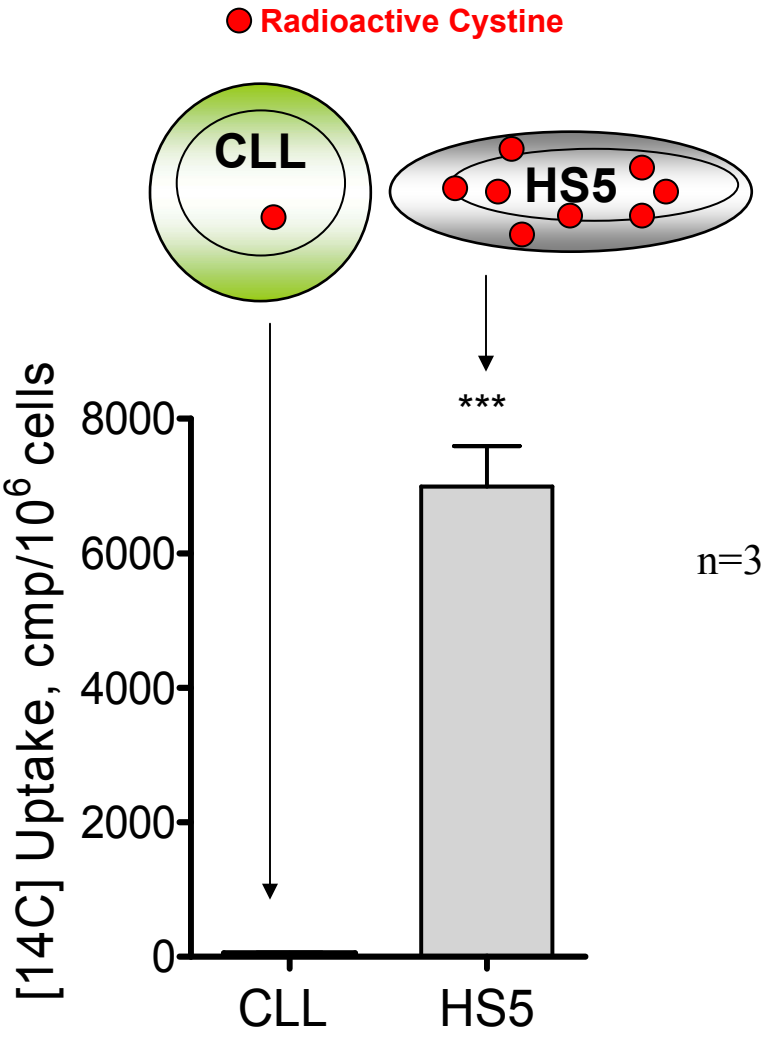
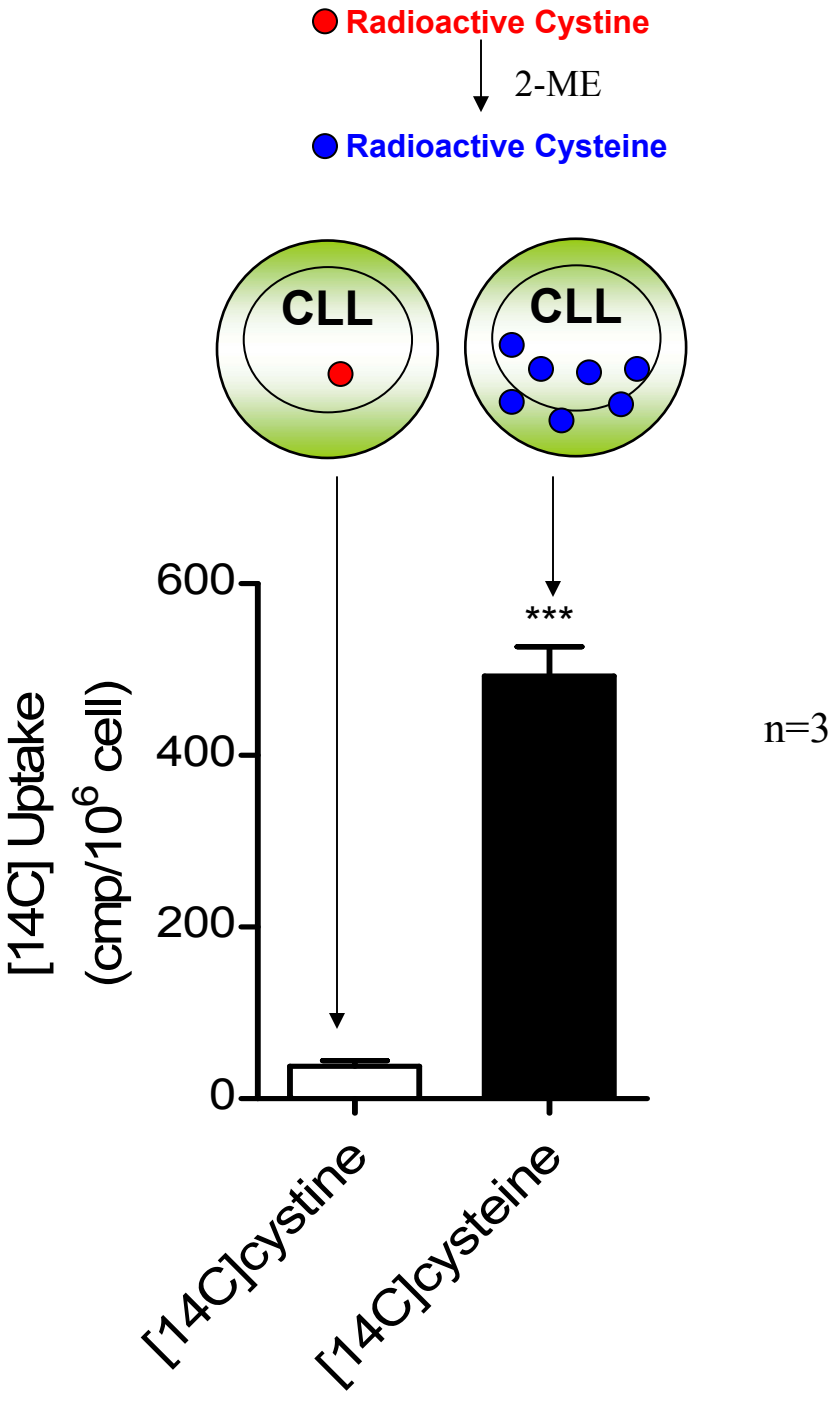

Radioactive Cystine
CLL
HS5
***
[14C] Uptake, cmp/10^6 cells
8000
6000
4000
2000
0
CLL
HS5
n=3

Figure 45. Comparison of uptake of cystine and cysteine by CLL cells. CLL were incubated in fresh cystine-free RPMI 1640 supplemented with dialyzed 10% FBS. [14C]-cystine and [14C]-cysteine were at (0.2 μCi/ml) and incubated for 4 hours. The cells were washed twice with cold PBS. Cell pellets were resuspended in 200 μL PBS, lysed with 3 mL scintillation fluid, and radioactivity was measured by a Beckman liquid scintillation counter. [14C]-Cysteine was generated by reduction of [14C]-cystine using 5 mM 2-mercaptoethanol (37°C, 15 min). Bar graph of mean ±SEM of 3 separate experiments using CLL cells from 3 different patients is shown (***, $p<0.001$).


Radioactive Cystine
2-ME
Radioactive Cysteine
CLL
CLL
600
400
200
0
[14C] Uptake
(cmp/10^6 cell)
***
[14C]cystine
[14C]cysteine

n=3

Figure 46. Increased radioactive uptake of CLL cells in the presence of HS5 stromal cells. CLL cultured alone or with HS5 stromal cells were incubated in fresh cystine-free RPMI 1640 supplemented with dialyzed 10% FBS. [14C]-cystine (PerkinElmer, Waltham, MA) was at (0.2 μCi/ml) and incubated for 6 h. The cells were washed twice with cold PBS. Cell pellets were resuspended in 200 μL PBS, lysed with 3 mL scintillation fluid, and radioactivity was measured by a Beckman liquid scintillation counter. Bar graph of mean ±SEM of 3 separate experiments using CLL cells from 3 different patients is shown (***, $p<0.001$).

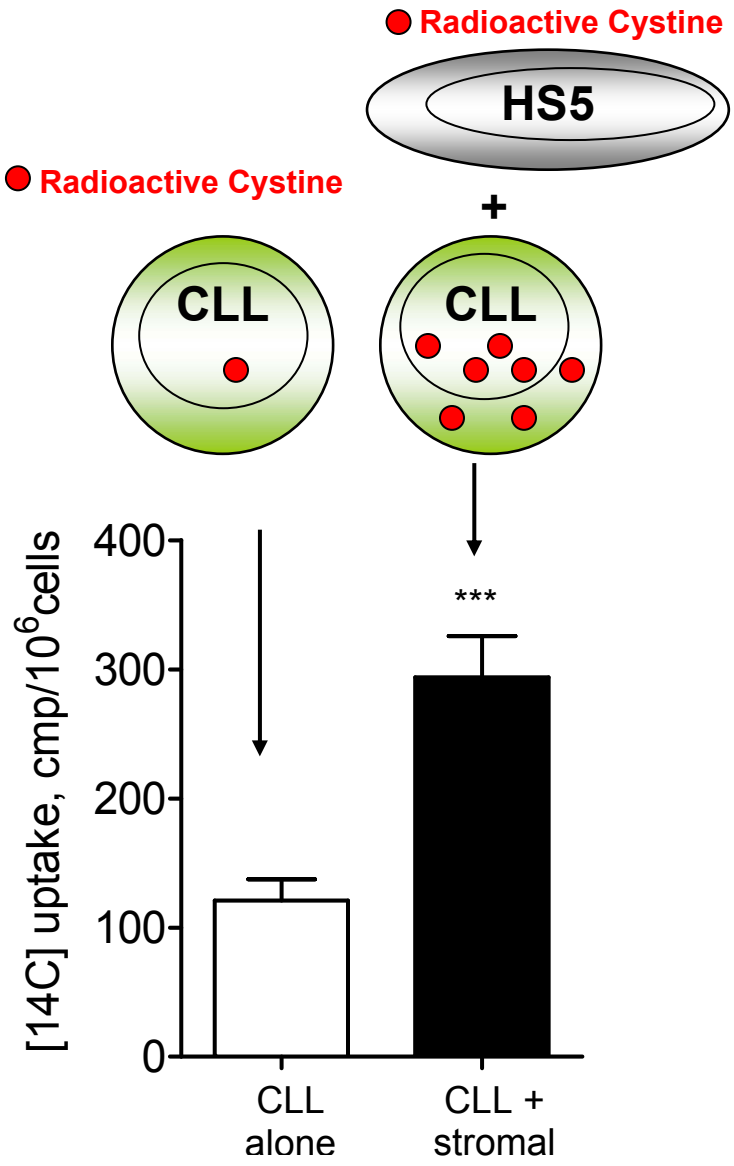
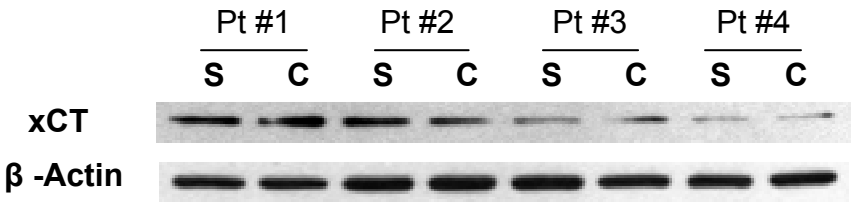

Radioactive Cystine
HS5
Radioactive Cystine
+
CLL
CLL
***
[14C] uptake, cmp/10^6cells
400
300
200
100
0
CLL alone
CLL + stromal

Figure 47. No change in expression of xCT in CLL cells co-cultured with HS5 stromal cells. CLL cells were cultured alone or with HS5 stromal cells for 3 days. Cell lysates of CLL cells (n=13) were prepared and equal amounts of protein were electrophoresed on SDS-PAGE gels using standard conditions. The proteins were transferred to nitrocellulose membranes, which were probed with the following antibodies: xCT (Novus Biologicals, Littleton, CO); actin (Sigma-Aldrich, St Louis, MO). Protein bands were visualized by chemiluminescent detection. The upper panel shows the representative western blot results, and the lower panel shows the quantitation of xCT band density of 13 CLL samples, using β-actin expression in the same sample as the internal control (mean ±SD, p=0.951; S, single culture of CLL cells alone; C, co-cultured with stromal cells).


Pt #1
Pt #2
Pt #3
Pt #4
S
C
S
C
S
C
S
C
xCT
β -Actin

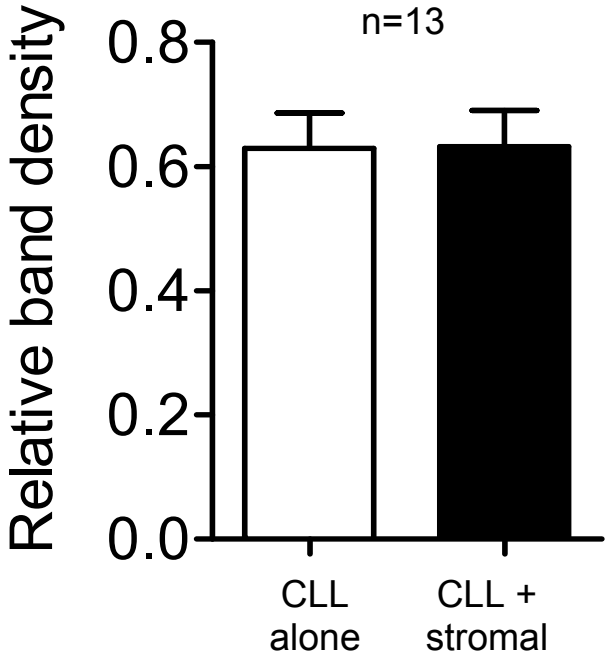

n=13
Relative band density
0.8
0.6
0.4
0.2
0.0
CLL alone
CLL + stromal

Figure 48. Extracellular cystine is required for stromal cells to enhance GSH synthesis in CLL cells. HS5 stromal cells was plated allowed to adhere for overnight and then the culture medium was removed, followed by a rinse with warm PBS. CLL cells were resuspended in cystine-free RPMI 1640 supplemented with 10% dialyzed FBS and added to the culture dish containing pre-washed HS5 stromal cells. 200 μM cystine was added to the single culture and cocultures as indicated. Cellular GSH level of CLL cells was measured on day 3. (n=3 patient samples; *, $p<0.05$; **, $p<0.01$).

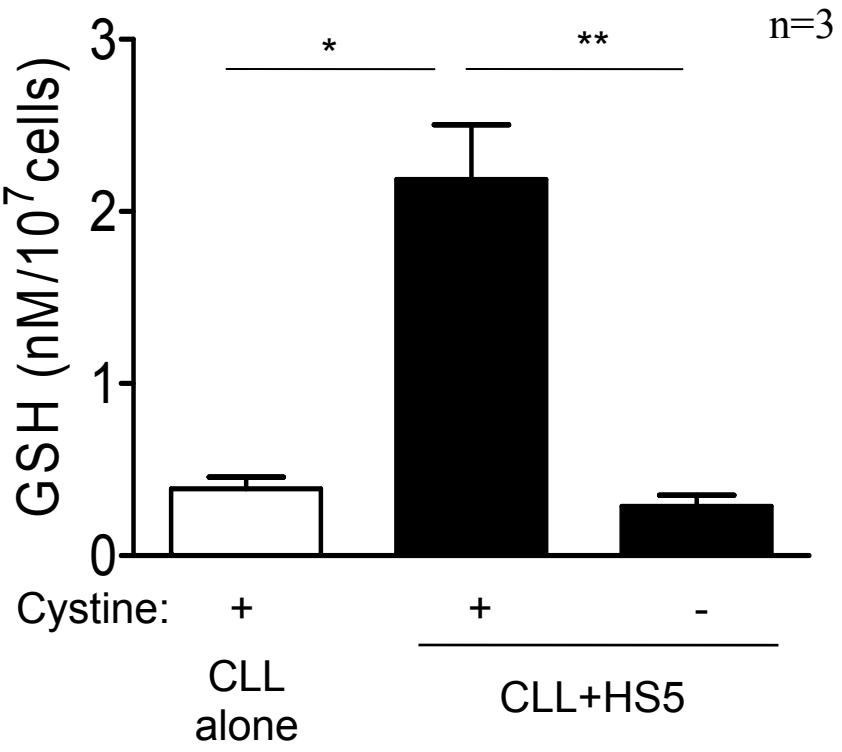

n=3
*
**
GSH (nM/10^7cells)
3
2
1
0
Cystine:
+
+
-
CLL alone
CLL+HS5

Figure 49. Stromal cells fail to protect CLL cells from drug-induced apoptosis in the absence of cystine. HS5 stromal cells was plated allowed to adhere for overnight and then the culture medium was removed, followed by a rinse with warm PBS. CLL cells were resuspended in cystine-free RPMI 1640 supplemented with 10% dialyzed FBS and added to the culture dish containing pre-washed HS5 stromal cells. 200 μM cystine was added to the single culture and cocultures as indicated. CLL cells were cultured alone or with a layer of HS5 stromal cells for 1 day and then incubated with 20 μM F-ara-A or 20 μM oxaliplatin for another 2 days. Cell viability was assessed by flow cytometry after annexin-V/PI staining (n=3 patient samples; *, $p<0.05$).

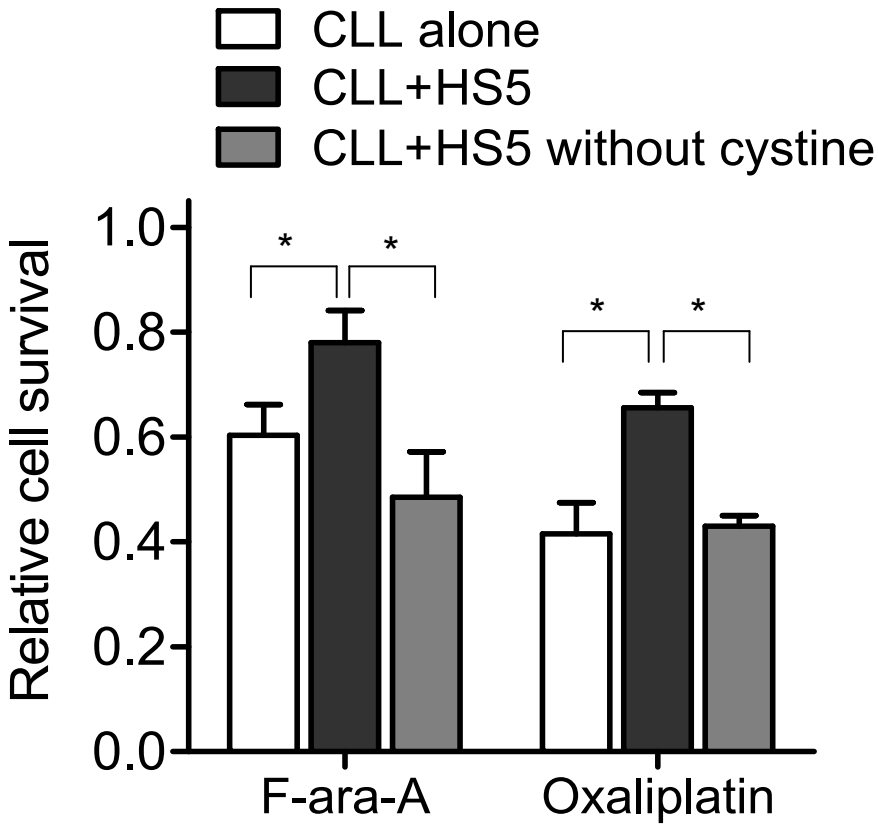
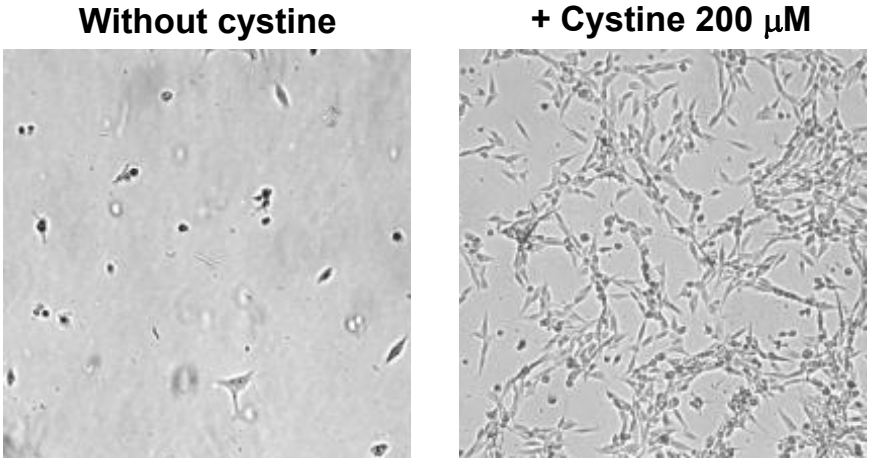

CLL alone
CLL+HS5
CLL+HS5 without cystine
Relative cell survival
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
*
F-ara-A
Oxaliplatin

Figure 50. Cell morphology and GSH contents of HS5 stromal cells in the presence and absence cystine. Most of HS5 cells lost viability and detached from the culture dish on day 3, and cellular GSH could not be determined due to loss of cells (indicated by *). HS5 stromal cells was plated allowed to adhere for overnight and then the culture medium was removed, followed by a rinse with warm PBS and incubated in medium with or with 200 μM cystine. HS5 cells were imaged in medium using a phase-contrast microscope with a 10X objective lens. Images were captured with a Nikon digital camera with the use of Camera Control Pro software (Nikon); when necessary, Adobe Photoshop 9.0 (Adobe Systems) was used for image processing. GSH assay and photographs were performed on day 4.


Without cystine
+ Cystine 200 μM

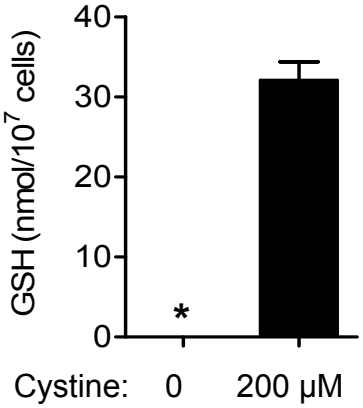
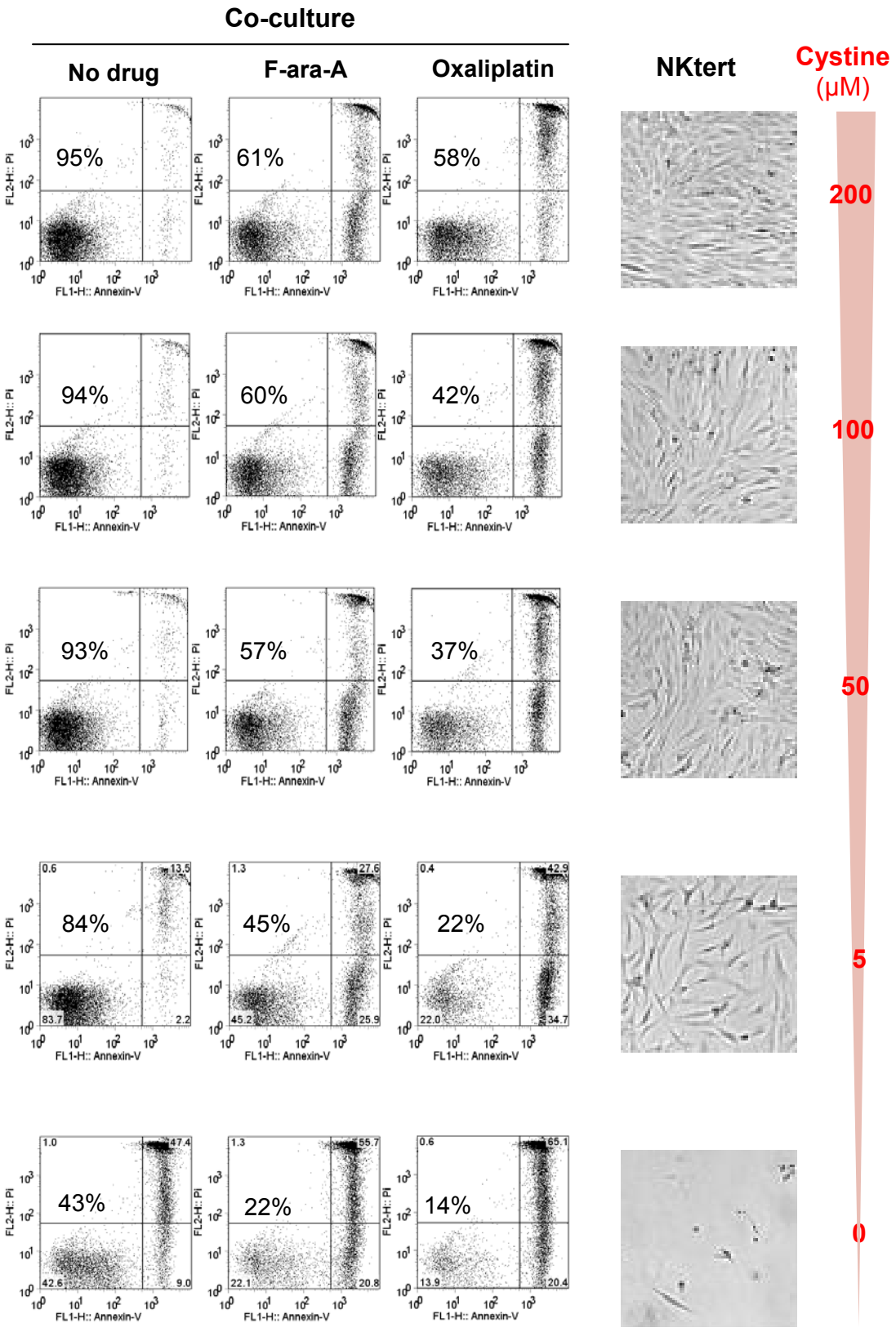
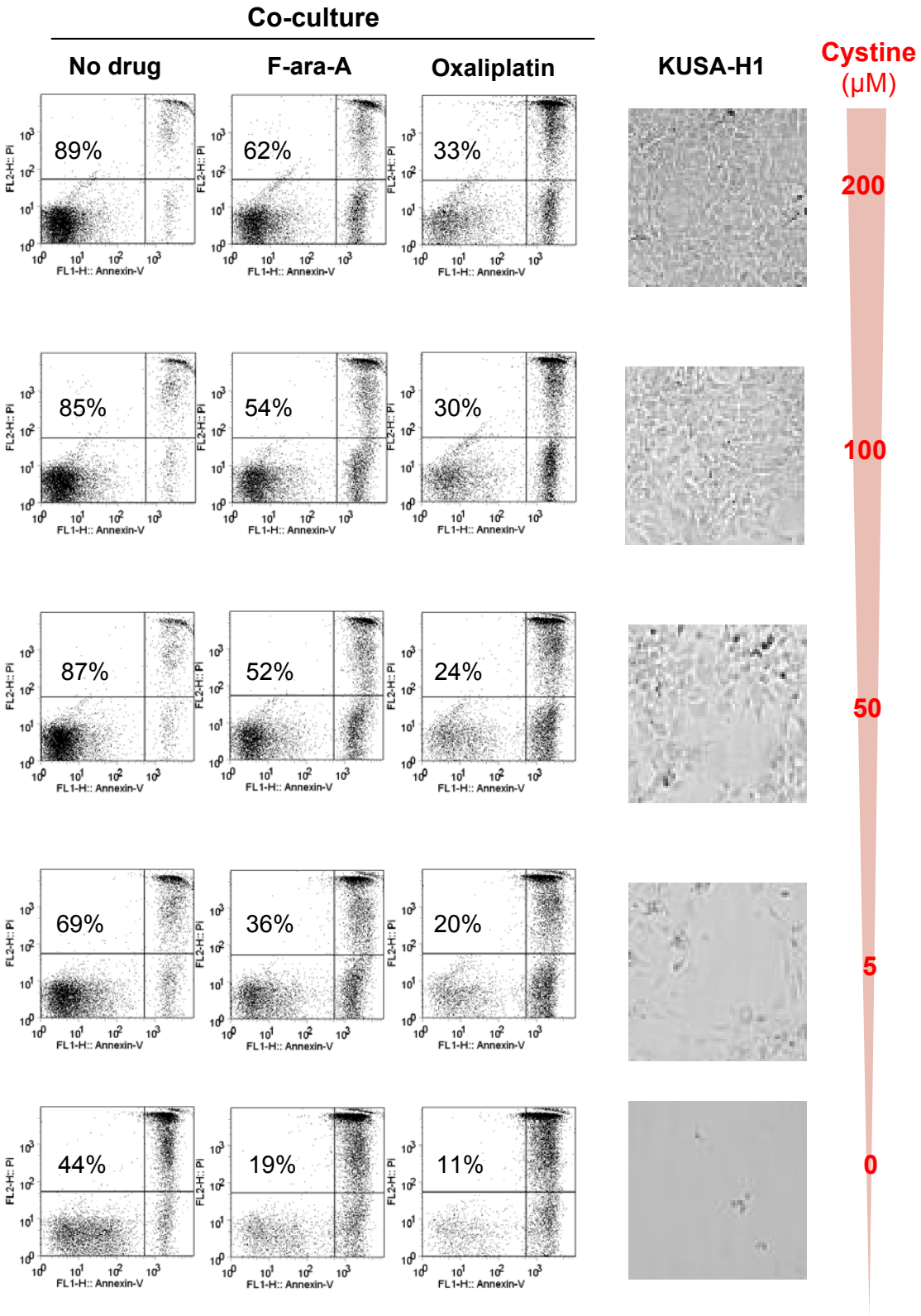

GSH (nmol/10^7 cells)
40
30
20
10
0
*
Cystine: 0
200 μM

Figure 51. Cystine-dependent protection of CLL cells by bone marrow stromal cells. The indicated concentrations of cystine were incubated with CLL cells alone or in co-culture with stromal cells (Nktert or KUSA-H1) for 24 h. The cells were then treated with F-ara-A (20 mM) or oxaliplatin (20 μM) for additional 48 h, and CLL cell viability was measured by flow cytometry analysis. The morphology of the stromal cells cultured with the indicated concentrations of cystine is also shown. Without stromal cells, the percent of viable CLL cells on day 3 was 50% without drug treatment, 22% with 20 μM F-ara-A treatment, and 13% with 20 μM oxaliplatin incubation.


Co-culture
No drug
F-ara-A
Oxaliplatin
NKtert
Cystine (μM)
200
100
50
5
0
95%
61%
58%
94%
60%
42%
93%
57%
37%
84%
45%
22%
43%
22%
14%
FL2-H:: PI
FL1-H:: Annexin-V


Co-culture
No drug
F-ara-A
Oxaliplatin
KUSA-H1
Cystine (μM)
89%
62%
33%
85%
54%
30%
87%
52%
24%
69%
36%
20%
44%
19%
11%
200
100
50
5
0
FL2-H:: PI
FL1-H:: Annexin-V

8. Targeting the biochemical pathway to circumvent drug resistance.

Since enhancement of GSH synthesis in CLL cells seemed to be a key biochemical mechanism by which bone marrow stromal cells promoted leukemia cell survival and drug resistance, I reasoned that abrogation of this protective mechanism would sensitize the leukemia cells to drug treatment in the stromal environment. One way to abolish this stromal protection mechanism would be to inhibit the cystine transporter Xc- to reduce their uptake of cystine so that its conversion to cysteine would be decreased. To avoid the non-specific impact of chemical inhibitors on cell viability, I used two different inhibitors of Xc- ((S)-4-carboxyphenylglycine [84] and sulfasalazine [122]) with a careful dose-titration to determine the concentrations that did not cause significant cytotoxicity, and then tested if such non-toxic concentrations of Xc- inhibitors could abrogate the stromal protective effect on CLL cells. As shown in **Fig 52**, HS5 cells reduced the sensitivity of CLL cells to F-ara-A or oxaliplatin, and addition of a subtoxic concentration of (S)-4-carboxyphenylglycine (500 μM) led to a substantial increase in F-ara-A or oxaliplatin-induced cytotoxicity, comparable to the cytotoxicity observed in CLL cells without stromal protection. Similar effect was consistently observed in a separate experiment using a different CLL patient sample co-cultured with another bone marrow cell line KUSA-H1. **Fig 53** shows that S-4-carboxyphenylglycine abolished the protective effect of KUSA-H1 stromal cells in a concentration-dependent manner, which is consistent with the result shown in **Fig 52**, where HS5 stromal cells were tested in a similar fashion using a different CLL patient sample. The ability of another cystine transporter inhibitor sulfasalazine (300 μM) to enhance drug-induced cytotoxicity was also observed in CLL cells co-cultured with stromal cells (**Fig 54**). I have also attempted to knockdown xCT

expression in the stromal cells by siRNA as an additional method to evaluate the role of Xc-. I tested 3 sets of xCT siRNA (from Invitrogen), but none of the siRNA was able to abolish xCT expression in the bone marrow stromal cells (**Fig 55**). I did not pursue this method further due to such technical difficulty. However, the important role of stromal Xc- in cystine uptake for conversion to cysteine to protect CLL cells is strongly supported by 3 lines of evidence: (1) two different Xc- inhibitors consistently abolished the stromal protection of CLL cells, (2) removal of cystine from the culture medium abrogated the ability of stromal cells to protect CLL cells, and (3) this protective effect could be rescued by adding exogenous cysteine (**Figs 39**).

Interestingly, suppression of the cysteine transporter ASC using a high concentration of serine (5 mM) as a competitive inhibitor induced only a moderate decrease of GSH in CLL cells (from 0.7 nmol/10^7 cells to 0.42 nmol/10^7 cells) during a 3-day co-culture with stromal cells (**Fig 56A**) and did not significantly affect their sensitivity to drug treatment (**Fig 56B**). These data suggest that in addition to ASC, CLL cells might also have other cysteine transporters such as Na^+-independent transporters (system-L), which could not be inhibited by serine [82, 123] (**Table 2**). The combination of cysteine competitors of ASC (serine, 5mM) and transporter A (a-methylamino-isobutyric acid (MeAIB)) still failed to circumvent drug resistance of CLL cells with stromal cells (**Fig 57**), indicating that there may be several cysteine transporters on CLL cells that responsible for cysteine uptake. This also explains why CLL cells have strong ability to uptake cysteine. I also tested another strategy to abrogate the stromal-mediated GSH protection of CLL cells by disabling the GSH antioxidant system in the cells. β-phenylethyl isothiocyanate (PEITC), a natural compound that can cause depletion of GSH and inhibition of glutathione

peroxidase [52], was used for this purpose. As shown in **Figs 58-59**, 5 μM PEITC, which by itself caused a depletion of GSH in CLL cells [52], induced about 50% loss of CLL cell viability, and substantially enhanced the cytotoxicity of F-ara-A (n = 30 samples) and of oxaliplatin (n=10) in CLL cells in the presence of HS5 stromal cells. I also examined the combinative effect of PEITC and F-ara-A or oxaliplatin on CLL cells in the presence of two other bone marrow stromal cell lines (StromaNKtert and KUSA-H1), and showed PEITC at 5 μM was also able to enhance the cytotoxic effect of F-ara-A or oxaliplatin (**Fig 60**). However, this concentration of PEITC was not as effective as that observed in the experiments with HS5, probably due to greater ability of StromaNKtert and KUSA-H1 to release cysteine into the medium compared to HS5 (**Fig 33**). When the concentration of PEITC was increased to 10 μM, this compound was highly effective in killing CLL cells, causing a loss of 72% and 54% cell viability in the presence of StromaNktert or KUSA-H1 cells, respectively (**Fig 61**). Combination of 10 μM PEITC and 20 μM oxaliplatin exhibited striking synergistic effect against CLL cells in the presence of StromaNKtert or KUSA-H1 stromal cells, leading to massive killing of CLL cells by more than 80% (**Fig 61**).

The loss of p53 in CLL cells due to deletion of chromosome 17p is known to confer significant resistance to standard chemotherapeutic agents [124, 125]. Indeed, primary CLL cells isolated from a patient with 17p deletion were highly resistance to F-ara-A and oxaliplatin regardless of bone marrow stromal cells (**Fig 62**). However, 10 μM PEITC was able to kill approximately 50% of the p53- CLL cells in the presence of KUSA-H1 stromal cells, and its combination with oxaliplatin was highly effective, resulting in more than additive cell killing with more than 80% loss of CLL cell viability (**Fig 62**). These

data together suggest that abrogation of the GSH antioxidant system is a potentially effective strategy to abolish the stromal protection of CLL cells *in vivo*.

Figure 52. Sensitization of CLL cells to F-ara-A and oxaliplatin in the presence of stromal cells by inhibition of cystine transport. CLL and HS5 cells in co-culture were first incubated with (S)-4-carboxyphenylglycine (S-4-CPG, 500 μM) for 24 h to inhibit cystine transport, and then exposed to F-ara-A (20 μM) or oxaliplatin (20 μM) for 48 h. Cell viability was analyzed by annexin-V/PI assay. Representative dot plots are shown with % viable cells (annexin V and PI double negative) indicated.

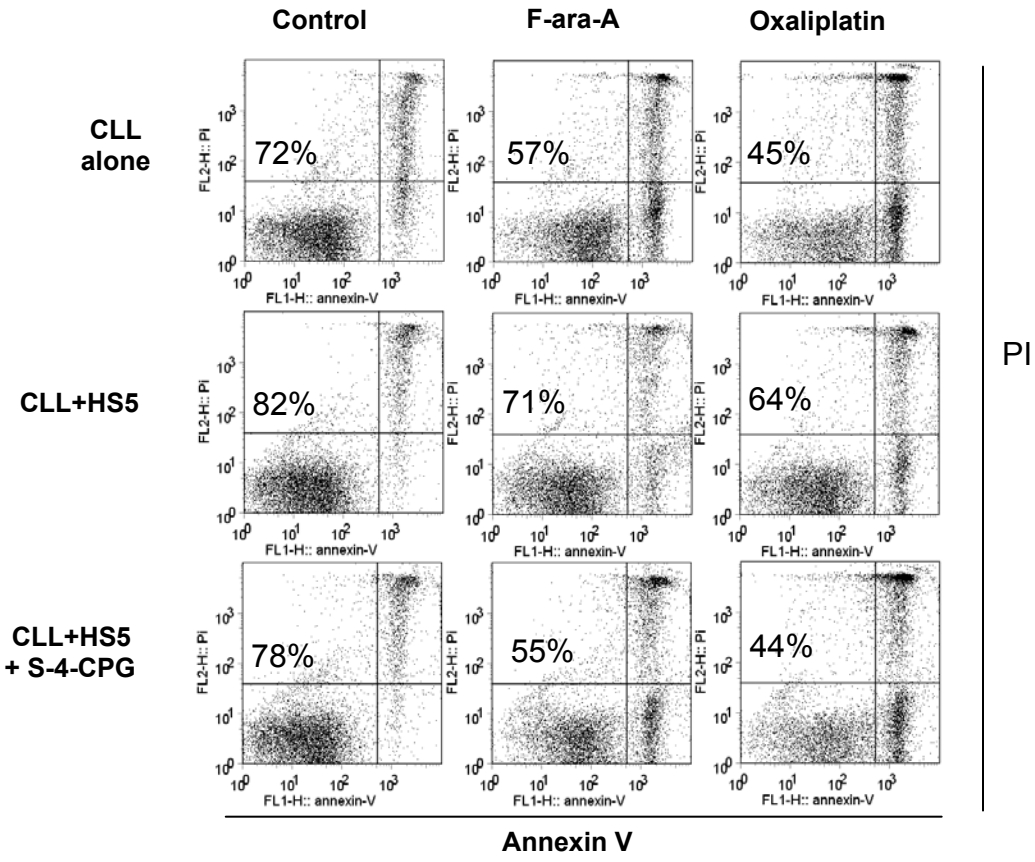

Control
F-ara-A
Oxaliplatin
CLL alone
CLL+HS5
CLL+HS5 + S-4-CPG
72%
57%
45%
82%
71%
64%
78%
55%
44%
FL2-H:: PI
FL1-H:: annexin-V
PI
Annexin V

Figure 53. Abrogation of the stromal protective effect on CLL cells by the Xc- inhibitor (S)-4-carboxyphenylglycine (S-4-CPG). CLL cells and stromal cells (KUSA-H1) in co-culture were first incubated with S-4-CPG (200, 500, and 1000 μM) for 24 h to inhibit cystine transport, and then exposed to F-ara-A (20 μM) or oxaliplatin (20 μM) for 48 h. Cell viability was analyzed by annexin-V/PI assay. The number in each panel indicates % of viable cells (annexin V and PI double negative).

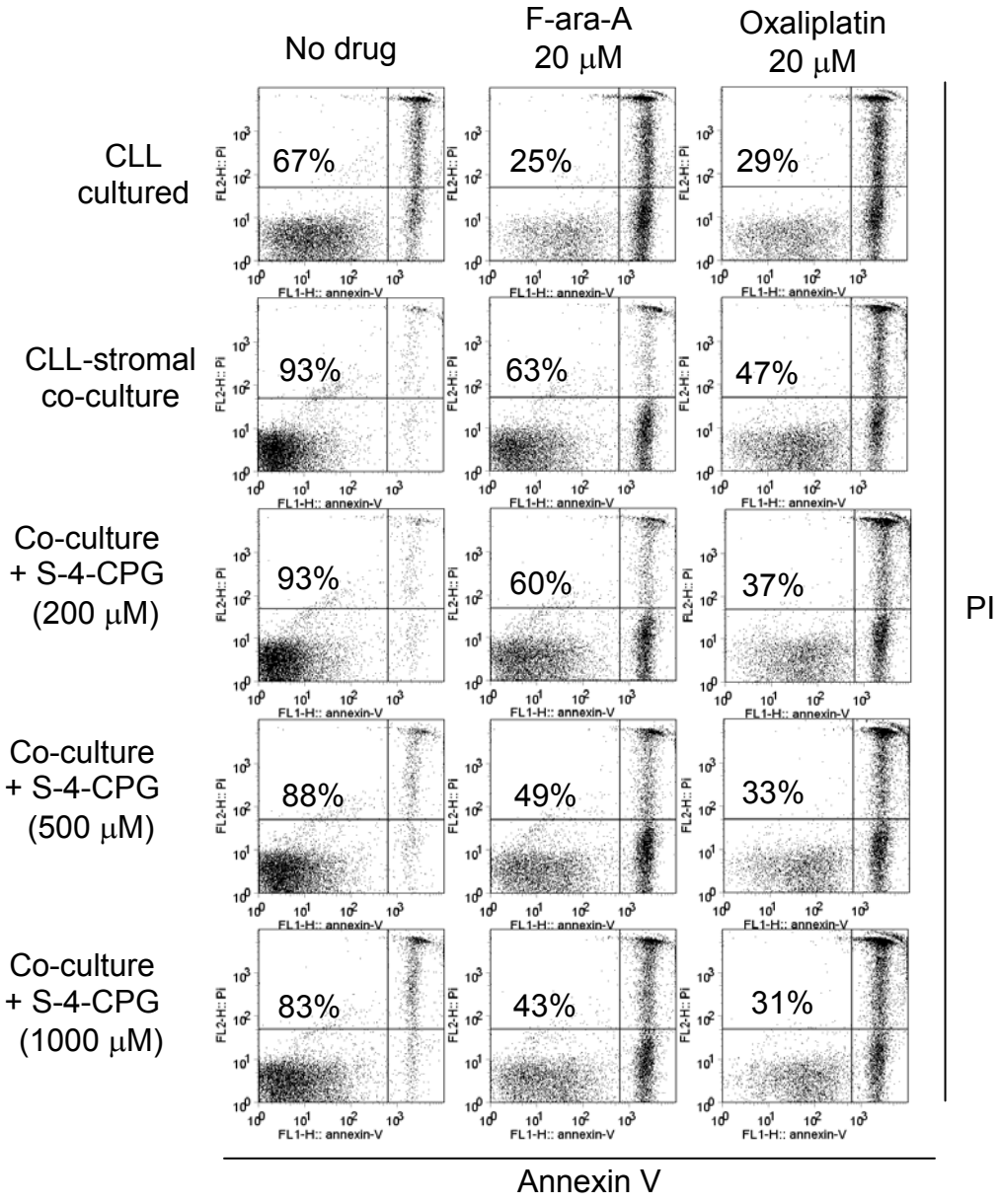

No drug
F-ara-A 20 μM
Oxaliplatin 20 μM
CLL cultured
CLL-stromal co-culture
Co-culture + S-4-CPG (200 μM)
Co-culture + S-4-CPG (500 μM)
Co-culture + S-4-CPG (1000 μM)
67%
25%
29%
93%
63%
47%
93%
60%
37%
88%
49%
33%
83%
43%
31%
FL2-H:: PI
FL1-H:: annexin-V
PI
Annexin V

Figure 54. Sensitization of CLL cells to F-ara-A and oxaliplatin by inhibition of cystine transport in the presence of stromal cells. CLL and stromal (KUSA-H1) cells in co-culture were first incubated with sulfasalazine (SAS, 300 μM) for 24 h to inhibit cystine transport, and then exposed to F-ara-A (20 μM) or oxaliplatin (20 μM) for 48 h. Cell viability was analyzed by flow cytometry after double staining with annexin-V and PI. Representative dot plots are shown with % viable cells (annexin V and PI double negative) indicated.

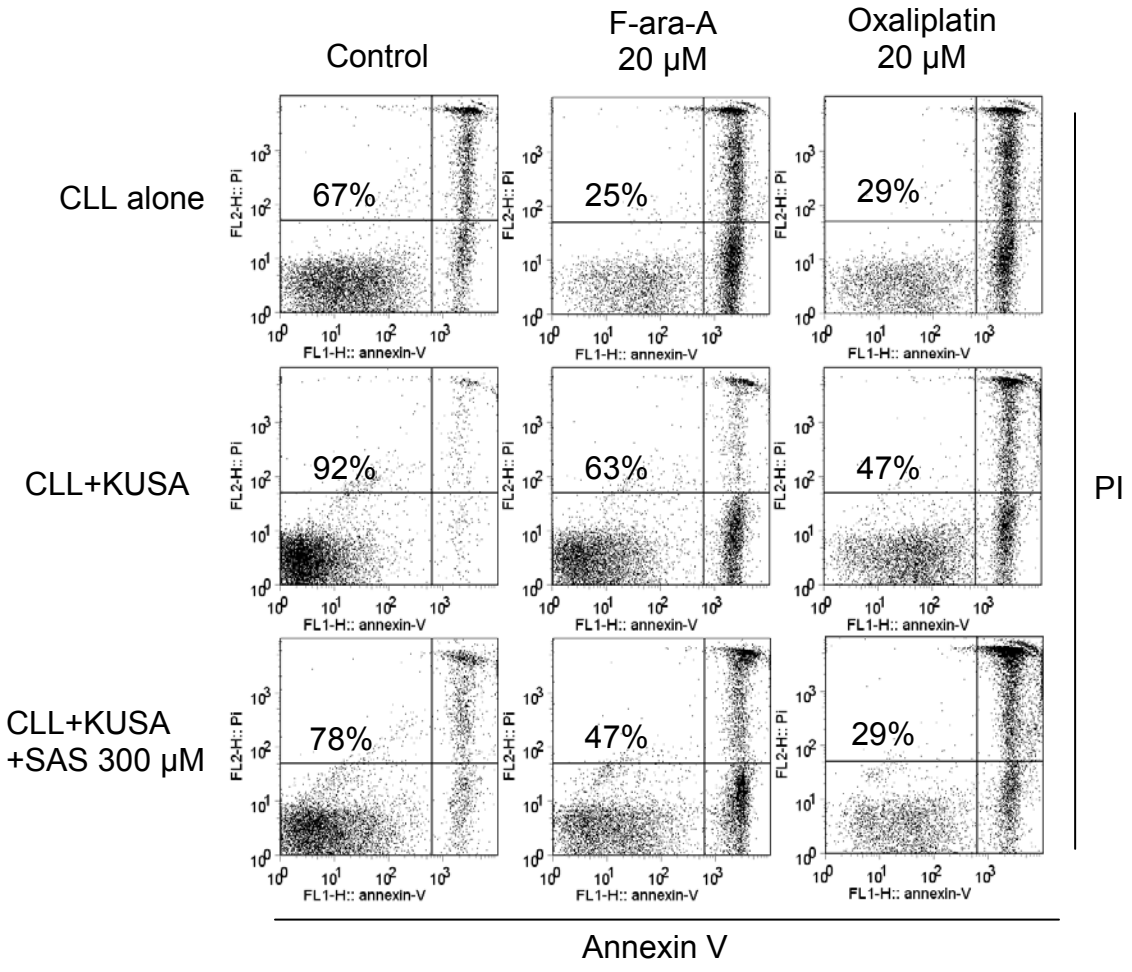

Control
F-ara-A
20 μM
Oxaliplatin
20 μM
CLL alone
CLL+KUSA
CLL+KUSA
+SAS 300 μM
67%
25%
29%
92%
63%
47%
78%
47%
29%
FL2-H:: PI
FL1-H:: annexin-V
PI
Annexin V

Figure 55. Unsuccessful knockdown of xCT expression by siRNA in bone marrow stromal cells. HS5 stromal cells were transfected with nonspecific, xCT RNA interference (RNAi; final concentrations, 20, 40, 100 nM) using Lipofectamine ™ 2000 transfection according to the manufacturer's instructions. The cells were then incubated in 24 well plates for 72 h prior to Western blot for xCT expression. RNAi with the same GC content as siRNA pools was used as a negative control.

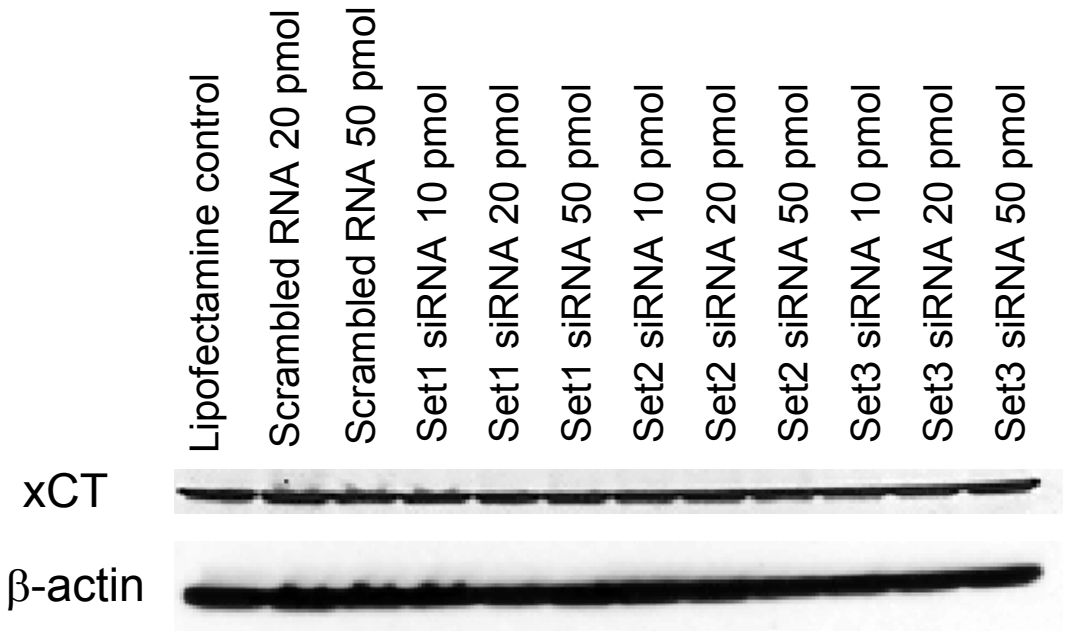
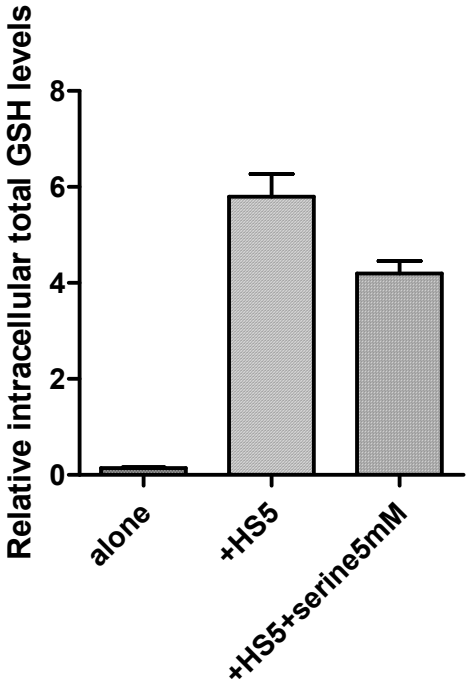

Lipofectamine control
Scrambled RNA 20 pmol
Scrambled RNA 50 pmol
Set1 siRNA 10 pmol
Set1 siRNA 20 pmol
Set1 siRNA 50 pmol
Set2 siRNA 10 pmol
Set2 siRNA 20 pmol
Set2 siRNA 50 pmol
Set3 siRNA 10 pmol
Set3 siRNA 20 pmol
Set3 siRNA 50 pmol
xCT
β-actin

Figure 56. The effect of serine on GSH level and drug resistance of CLL cells in the presence of HS5 stromal cells. **(A)** Comparison of cellular GSH level of CLL cells cultured alone and co-cultured with HS5 stromal cells in the presence or absence of exogenous serine. Serine was added at the beginning of co-culture. GSH assay was preformed on day 3. (**B**) Serine has no effect on drug resistance of CLL cells co-cultured with HS5 stromal cell. CLL and HS5 stromal cells in co-culture were first incubated with 5mM serine, and then exposed to F-ara-A (20 μM) or oxaliplatin (20 μM) for 48 h. Cell viability was analyzed by flow cytometry after double staining with annexin-V and PI. Representative dot plots are shown with % viable cells (annexin V and PI double negative) indicated.

A


Relative intracellular total GSH levels
8
6
4
2
0
alone
+HS5
+HS5+serine5mM

B

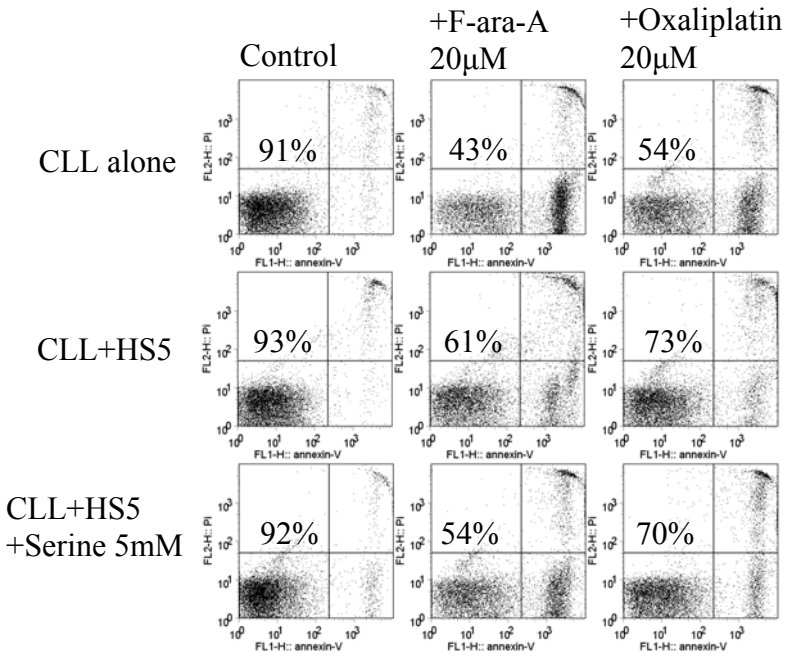

Control
+F-ara-A 20µM
+Oxaliplatin 20µM
CLL alone
CLL+HS5
CLL+HS5 +Serine 5mM
91%
43%
54%
93%
61%
73%
92%
54%
70%
FL2-H:: PI
FL1-H:: annexin-V

Table 2. Amino acid transport systems of mammalian cells. Transmembrane amino acid transport is catalysed by a number of discrete systems. C: cysteine (Adapted from: *Hyde R, Taylor PM, Hundal HS. The Biochemical journal 2003; 373: 1-18*).

(ai) Neutral-amino-acid transporters: sodium-dependent

System	Protein	Gene	Amino acid substrates (one-letter code)	Notes
A	SAT1	SLC38A1	G, A, S, C, Q, N, H, M, T, Me-AIB, P, Y, V	Short-chained-neutral-amino-acid transport.
	SAT2	SLC38A2	G, P, A, S, C, Q, N, H, M, Me-AIB	Sensitive to low pH. Ubiquitous expression.
	SAT3	SLC38A4	G, P, A, S, C, N, M, H, K, R	SAT3 may also function as a Na^+-independent cationic amino acid transporter.
ASC	ASCT1	SLC1A4	A, S, C	High-affinity short-chain-amino-acid exchanger.
	ASCT2	SLC1A5	A, S, C, T, Q	Ubiquitous expression.
B^0	ASCT2	SLC1A5	A, S, C, T, Q, F, W, Y	Broad substrate specificity. Expressed on apical surface of many epithelia. May include novel isoforms of ASCT2 (e.g. a novel gene recently characterized by Pollard et al. [216]).
BETA	GAT1	SLC6A1	GABA	Widely expressed Cl^--dependent GABA, betaine and taurine transporters.
	GAT2	SLC6A13	GABA, betaine, P, β-A	
	GAT3	SLC6A11	GABA, betaine, taurine	
	BGT1	SLC6A12	GABA, betaine	
	TAUT	SLC6A6	Taurine	
Gly	GLYT1	SLC6A9	G, sarcosine	Na^+- and Cl^--dependent high-affinity glycine transport.
	GLYT2	SLC6A5	G, sarcosine	Expressed in brain and some non-neural tissues.
IMINO	–	–	P	Na^+-dependent epithelial proline transporter, inhibited by Me-AIB.
N	SN1	SLC38A3	Q, N, H	Li^+-tolerant transport of Gln, Asn and His. H^+ antiport.
	SN2	SLC38A5	Q, N, H, S, G	Li^+-intolerant variants described
N^m	–	–	Q, N, H	
N^b	–	–	Q, N, H	
PHE	–	–	F, M	Brush-border transporter for Phe and Met
PROT	PROT	SLC6A7	P	Proline transporter in central nervous system.

(aii) Neutral-amino-acid transporters: sodium-independent

System	Protein	Gene	Amino acid substrates	Notes
asc*	Asc1	SLC7A10	G, A, S, C, T	Small neutral AA exchanger.
	Asc2		G, A, S, T	
imino	PAT1/LYAAT1	SLC36A1	P, G, A, β-A, GABA Me-AIB	H^+-coupled transport of small neutral amino acids. Inhibited by Me-AIB.
	PAT2/LYAAT2	SLC36A2	P, G, A, β-A, GABA Me-AIB	
L*	LAT1	SLC7A5	H, M, L, I, V, F, Y, W, Q	Ubiquitously expressed exchanger for large hydrophobic amino acids.
	LAT2	SLC7A8	A, S, C, T, N, Q, H, M, L, I, V, F, Y, W	
T	TAT1	SLC16A10	F, Y, W	Aromatic-amino-acid transporter. H^+/monocarboxylate transporter family – insensitive to pH, however.

(bi) Anionic-amino-acid transporters: sodium-independent

System	Protein	Gene	Amino acid substrates	Notes
X^-_{AG}	EAAT1	SLC1A3	E, D	Widespread Glu and Asp transporter. K^+ antiport.
	EAAT2	SLC1A2	E, D	Substrate-dependent uncoupled anion flux.
	EAAT3	SLC1A1	E, D, C	Lack of stereospecificity toward Asp.
	EAAT4	SLC1A6	E, D	
	EAAT5	SLC1A7	E, D	

(bii) Anionic-amino-acid transporters: sodium-independent

System	Protein	Gene	Amino acid substrates	Notes
x^{-c*}	xCT	SLC7A11	E, Ci	Electroneutral Glu/cystine exchanger.
–	XAT2	–	D, E	Non-functional upon 4F2hc/rbAT heavy-chain co-expression. Predicted to associate with a novel glycoprotein.

(ci) Cationic-amino-acid transporters: sodium-dependent

System	Protein	Gene	Amino acid substrates	Notes
$B^{0,+}$	ATB(o,+)	SLC6A14	K, R, A, S, C, T, N, Q, H, M, I, L, V, F, Y, W	Blastocysts and possibly brush-border membrane. Broad specificity for neutral and cationic amino acids. Accepts BCH.
y^+L^*	y^+LAT1	SLC7A7	K, R, Q, H, M, L	Na^+-dependent cationic/neutral-amino-acid exchanger. Electroneutral.
	y^+LAT2	SLC7A6	K, R, Q, H, M, L, A, C	

(cii) Cationic-amino-acid transporters: sodium-independent

System	Protein	Gene	Amino acid substrates	Notes
$b^{0,+**}$	b(o,+)AT	SLC7A9	K, R, A, S, C, T, N, Q, H, M, I, L, V, F, Y, W, Ci	Broad-specificity cationic- and neutral-amino-acid exchanger.
y^+	Cat-1	SLC7A1	R, K, H	Cationic-amino-acid (and Na^+-dependent neutral-amino-acid) transport. Variable degree of *trans*-stimulation.
	Cat-2	SLC7A2	R, K, H	
	Cat-3	SLC7A3	R, K	
	Cat-4	SLC7A4	Unknown	

F**igure 57. The combination of inhibitors of ASC and transporter A fails to circumvent drug resistance of CLL cells in the presence of HS5 stromal cell.** CLL and HS5 stromal cells in co-culture were first incubated with 5mM serine and 5mM a-methylamino-isobutyric acid (MeAIB), and then exposed to F-ara-A (20 μM) or oxaliplatin (20 μM) for 48 h. Cell viability was analyzed by flow cytometry after double staining with annexin-V and PI. Representative dot plots are shown with % viable cells (annexin V and PI double negative) indicated.

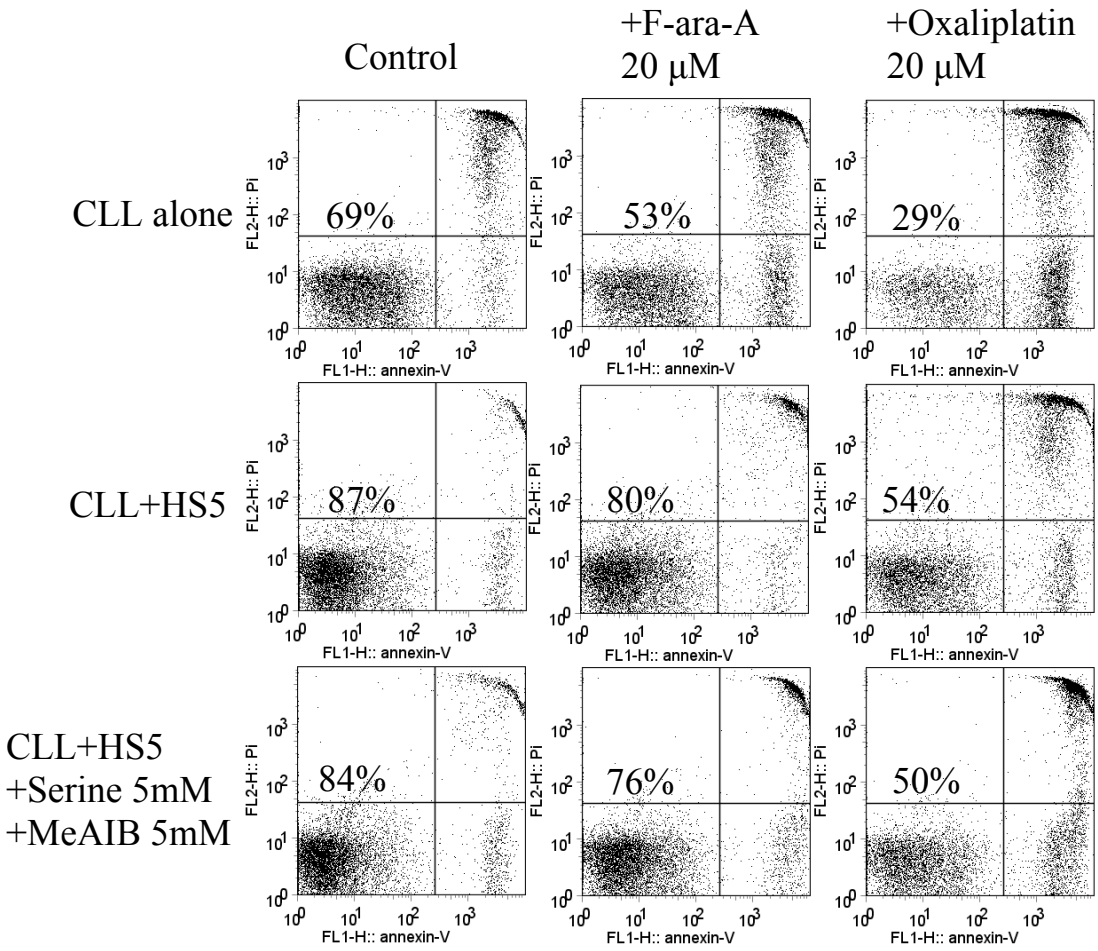

Control
+F-ara-A
20 µM
+Oxaliplatin
20 µM
CLL alone
CLL+HS5
CLL+HS5
+Serine 5mM
+MeAIB 5mM
69%
53%
29%
87%
80%
54%
84%
76%
50%
FL2-H:: PI
FL1-H:: annexin-V

Figure 58. Enhancement of cytotoxicity by the GSH-depleting agent PEITC in CLL cells co-cultured with stromal cells. CLL cells were cultured alone or with a layer of HS5 stromal cells for 1 day, and then treated with 20 μM F-ara-A, 20 μM oxaliplatin for another 48 hours, 5 μM PEITC for 5 hours, or their combination as indicated. Cell viability was measured by flow cytometry analysis after staining with annexin V/PI. The representative dot plot is shown. The number in each dot blot indicates % of viable cells (annexin V/PI double negative).

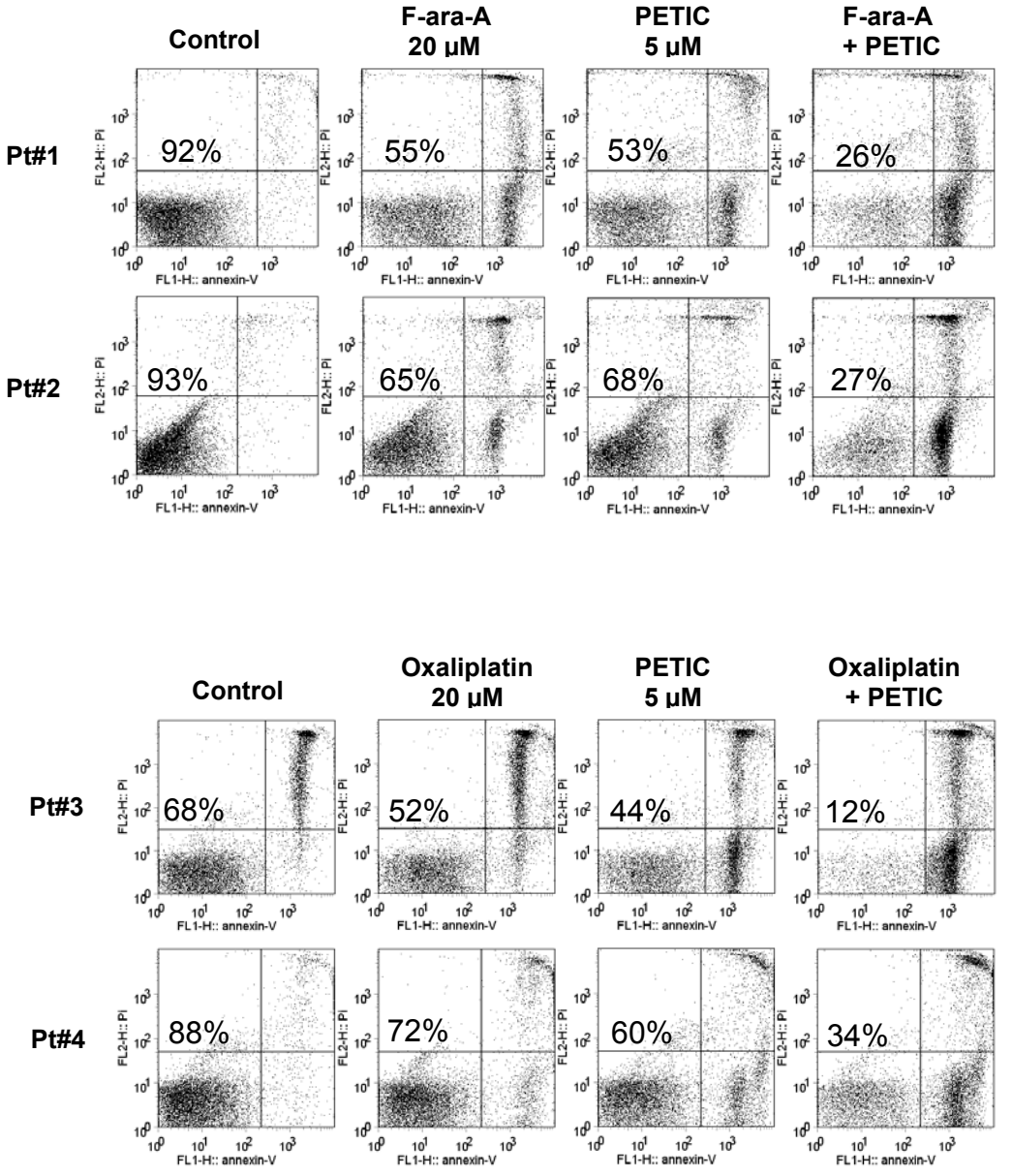

Control
F-ara-A
20 μM
PETIC
5 μM
F-ara-A
+ PETIC
Pt#1
92%
55%
53%
26%
Pt#2
93%
65%
68%
27%
Control
Oxaliplatin
20 μM
PETIC
5 μM
Oxaliplatin
+ PETIC
Pt#3
68%
52%
44%
12%
Pt#4
88%
72%
60%
34%
FL2-H:: PI
FL1-H:: annexin-V
Annexin V

Figure 59. Effect of PEITC (5 μM) on CLL cell viability cultured alone or with HS5 cells in the presence or absence of F-ara-A or oxaliplatin. CLL cells were cultured alone or with a layer of HS5 stromal cells for 1 day, and then treated with 20 μM F-ara-A, 20 μM oxaliplatin for another 48 hours, 5 μM PEITC for 5 hours, or their combination as indicated. Cell viability was measured by flow cytometry analysis after staining with annexin V/PI. The bar graph shows the mean ±SEM of separate experiments using multiple CLL patient samples.

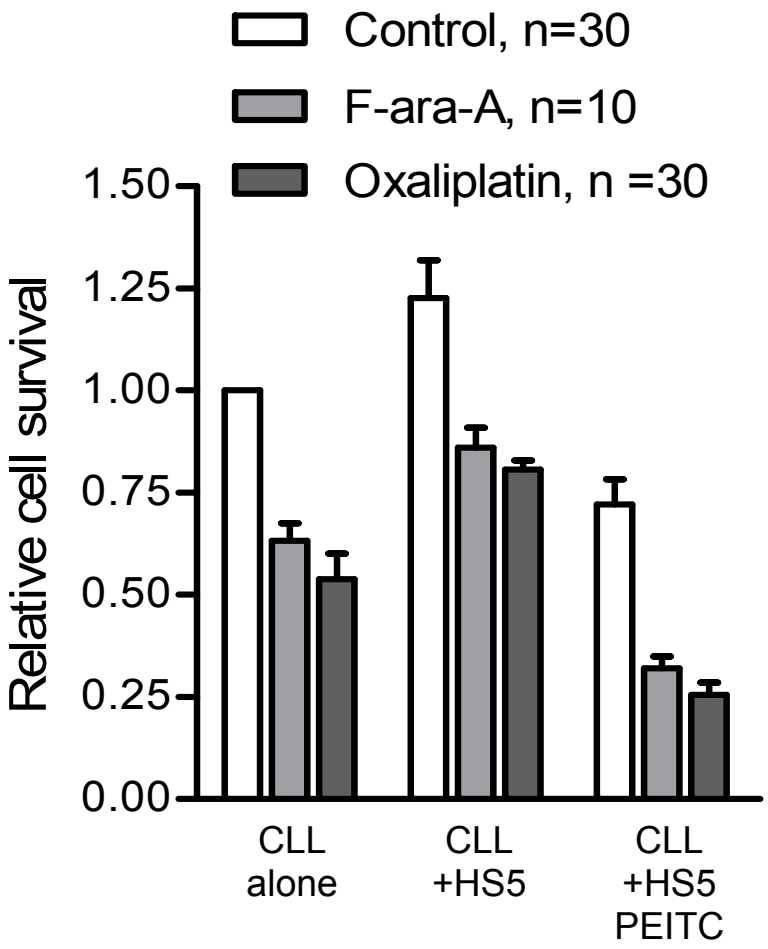
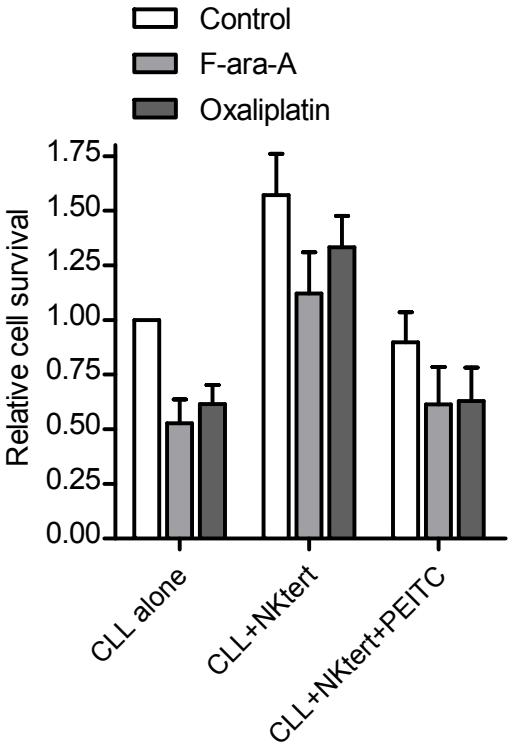

Control, n=30
F-ara-A, n=10
Oxaliplatin, n =30
Relative cell survival
1.50
1.25
1.00
0.75
0.50
0.25
0.00
CLL alone
CLL +HS5
CLL +HS5 PEITC

Figure 60. Effect of PEITC (5 μM) on CLL cell viability cultured alone or with StromaNktert (A) or KUSA-H1 (B) stromal cells in the presence or absence of F-ara-A or oxaliplatin. CLL cells were cultured alone or with a layer of StromaNKtert or KUSA-H1 stromal cells for 1 day, and then treated with 20 μM F-ara-A, 20 μM oxaliplatin for another 48 hours, 5 μM PEITC for 5 hours, or their combination as indicated. Cell viability was measured by flow cytometry analysis after staining with annexin V/PI. The bar graph shows the mean ±SEM of separate experiments using 6 different CLL samples.

A


Control
F-ara-A
Oxaliplatin
Relative cell survival
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
CLL alone
CLL+NKtert
CLL+NKtert+PEITC

B

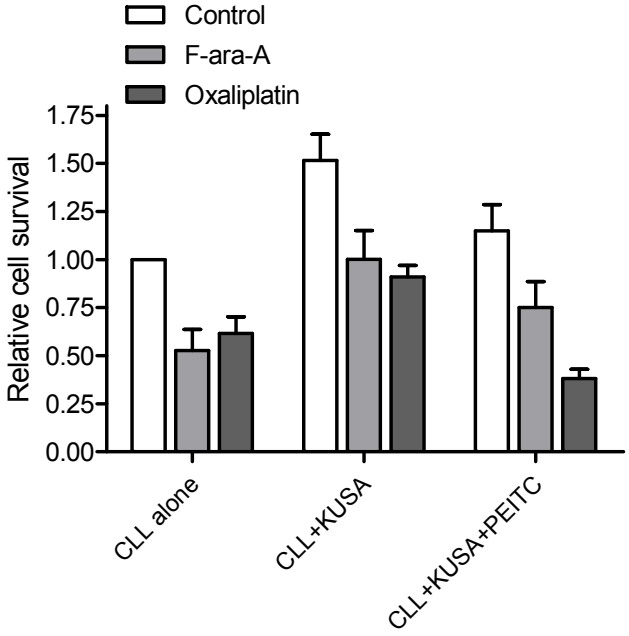

Control
F-ara-A
Oxaliplatin
Relative cell survival
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
CLL alone
CLL+KUSA
CLL+KUSA+PEITC

Figure 61. Synergistic effect of PETIC (10 μM) and oxaliplatin (20 μM) in killing CLL cells co-cultured with StromaNktert or KUSA-H1 stromal cells. CLL cells were cultured alone or with a layer of KUSA-H1 stromal cells for 1 day, and then treated with 20 μM F-ara-A, 20 μM oxaliplatin for another 48 hours, 10 μM PEITC for 5 hours, or their combination as indicated. Cell viability was measured by flow cytometry analysis after staining with annexin V/PI. The representative dot plot is shown. The number in each dot blot indicates % of viable cells (annexin V/PI double negative).

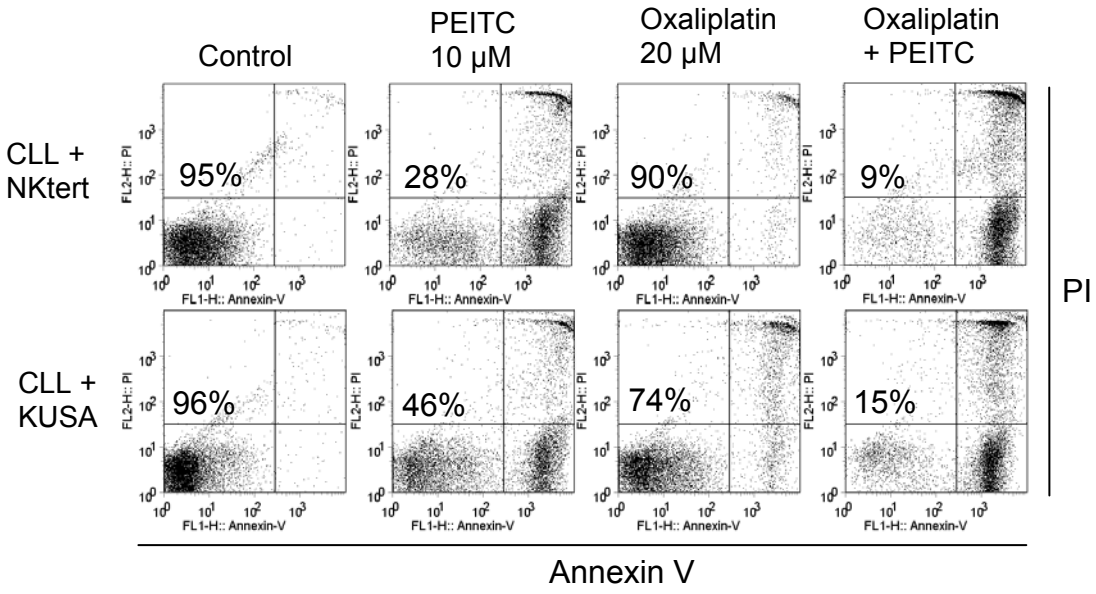
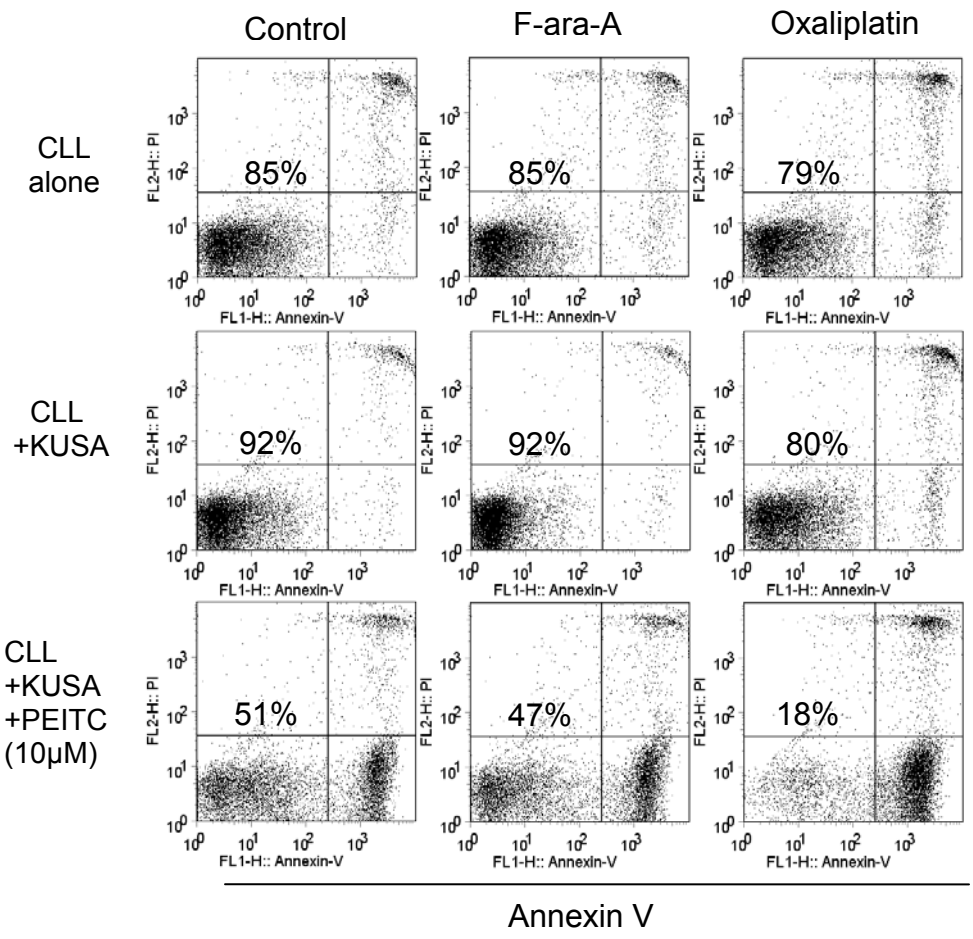

Control
PEITC
10 μM
Oxaliplatin
20 μM
Oxaliplatin
+ PEITC
CLL +
NKtert
CLL +
KUSA
95%
28%
90%
9%
96%
46%
74%
15%
FL2-H:: PI
FL1-H:: Annexin-V
PI
Annexin V

Figure 62. PEITC (10 μM) was effective in killing CLL cells with p53 deletion (17p-) and enhance the activity of F-ara-A (20 μM) or oxaliplatin (20 μM) in the presence of stromal cells (KUSA-H1). CLL cells were cultured alone or with a layer of KUSA-H1 stromal cells for 1 day, and then treated with 20 μM F-ara-A, 20 μM oxaliplatin for another 48 hours, 5 μM PEITC for 5 hours, or their combination as indicated. Cell viability was measured by flow cytometry analysis after staining with annexin V/PI. The representative dot plot is shown. The number in each dot blot indicates % of viable cells (annexin V/PI double negative).

CLL cells with 17p deletion



DISCUSSION

1. Stromal-mediated GSH upregulation in CLL cells maintains redox balance and promotes survival.

CLL is the most common adult leukemia characterized by abnormal accumulation of functionally defective B-lymphocytes in the blood, bone marrow, spleen, and other organs, and eventually leads to functional failure and patient death [1, 113, 126]. The accumulation of CLL cells *in vivo* is due in part to a prolonged CLL cell survival or reduced apoptosis [108]. Paradoxically, CLL cells are known to have high levels of oxidative stress [49, 52, 127] and often exhibit spontaneous apoptosis *in vitro* under regular culture conditions [128]. These observations suggest that the tissue environment may promote CLL cell survival *in vivo*. Certain soluble stromal factors and the direct cell-cell contact between CLL and stromal cells have been suggested to contribute to CLL cell survival and drug resistance [99, 129, 130]. However, at the biochemical level, how stromal cells communicate with CLL cells to promote their survival largely remains unknown.

Compared to normal lymphocytes, CLL cells exhibit increased production of reactive oxygen species (ROS) and are under oxidative stress [49-51]. This has been further proven by the fact that CLL cells are quite sensitive to ROS-mediated anticancer agents [49, 52]. Moreover, B-CLL cells are more susceptible to H_2O_2 than normal lymphocytes [53]. All these indicate that CLL cells are highly dependent on anti-oxidant system to maintain redox balance. Glutathione (GSH), the chief non-protein intracellular sulfhydryl, is the major antioxidant that maintains a redox balance in the cellular compartments. Besides removing endogenous free radical, increased GSH levels largely affect the efficacy and interactions of a variety of antineoplastic interventions. While GSH is so important, CLL

cells seem not be able to maintain GSH by itself. An interesting report showed that, when CLL cells were cultured *in vitro*, there was a significantly rapid decrease in cellular GSH concomitant with spontaneous apoptosis of CLL cells [97]. The rapid GSH depletion was not observed with the T cells from CLL patients or with either B or T cells from normal subjects indicating that this phenomenon is unique to CLL cells. In this study, I also found that CLL cells depleted cellular GSH quickly within 3 days. Interestingly, CLL maintained GSH level in the presence of a layer of bone marrow stromal cells (**Figs 8-9**). This is accompanied by decreased ROS level, enhanced cell survival, and resistance to exogenous ROS stress (**Figs 10-11**). All these data suggest that the *in vivo* microenvironment could enhance GSH level in CLL cells thus maintain redox balance of CLL cells and protect cells from spontaneous apoptosis.

GSH is the most abundant antioxidant involved in not only redox balance but also cell survival and drug resistance [71, 73]. Increasing GSH in CLL cells by either GSH or its precursor NAC protects CLL cells for a long term survival *in vitro* without stromal cells (**Figs 15-16**); while decreasing GSH in CLL cells co-cultured with stromal cells by PEITC circumvents microenvironment-induced drug resistance (**Fig 20**). All these suggest that stromal-upregulated GSH in CLL cells plays a key role in mediating stromal protection of CLL cells from spontaneous and drug-induced apoptosis.

2. The low-molecular-weight fraction of the stromal medium is the survival factor.

The interaction of cancer cells with stromal cells is mediated by cell contact and soluble factors. In my study, I found that the HS5 stromal cells increased GSH in CLL cells and protected CLL cells for survival regardless of cell contact (**Fig 21**), suggesting that the stromal-secreted soluble factors mediate GSH upregulation in CLL cells.

However, this study does not exclude the protective role of cell contact in CLL cells. In other co-culture systems including bone marrow stromal cell lines StromaNKtert or KUSA-H1, I observed enhanced protective effect in the direct co-culture of stromal cells and CLL cells (**Fig 22**). It is likely that HS5 stromal cells lack certain adhesion molecules, or secrete soluble form of adhesion molecules that block cell adhesion mediated by cell surface form of that adhesion molecules. The greater protection enhanced by cell contact suggests that the direct contact between stromal and CLL cells also contribute to the overall protective effect of stromal cells. The important role of soluble factor is further confirmed using the conditioned medium from HS5 stromal cell culture (**Fig 24**).

Stromal cells secrete a variety of soluble factors. Firstly I examined the relative levels of the cytokines and chemokines secreted in the conditioned medium of stromal cells. It has been known that the cell interaction could induce tremendous change of secreted cytokines and chemokines [131]. However, I did not observe any difference of secreting pattern in the medium of single HS5 cell cultures and CLL/HS5 cell co-cultures. By sorting stromal conditioned medium to high molecular weight fraction including cytokines, chemokines, etc. and low molecular weight fraction including amino acid, lipid, etc., interestingly, I found that it was the low molecular weight fraction that contributed to the maintenance of cellular GSH pool and protected CLL cells from spontaneous and drug-induced apoptosis (**Figs 26-27**). Failure to detect GSH in the culture medium of stromal cells indicates that the low molecular weight mediator is not GSH, and direct release of GSH into the medium by HS5 stromal cells was unlikely the major mechanism by which the stromal cells enhanced GSH in CLL cells. Undetectable

GSH in medium also suggests that the difference of GSH level in CLL cells with and without stromal cells was not due to difference of GSH depletion but caused by the difference of GSH synthesis. GSH synthesis is a two-step enzymatic process involving the ligation of glutamate with cysteine to form γ-glutamylcysteine and the addition of glycine to the C-terminal of γ -glutamylcysteine to form glutathione tripeptide. The synthesis of γ -glutamylcysteine is a rate-limiting step catalyzed by γ-glutamylcysteine ligase (GCL) [116]. Increased expression of the catalytic subunit (GCLC) is correlated with elevated GSH levels and drug resistance in tumor cells [74, 75]. Another important rate-limiting factor in GSH synthesis is the availability of the substrate cysteine, whose cellular concentration approximates the Km value of GCLC [76]. My study showed that GCLC expression was readily detected in CLL cells, and that the presence of stromal cells did not enhance the enzyme expression (**Fig 32**). Thus GCLC expression is unlikely a limiting factor in CLL cells, and the stroma-induced increase in GSH synthesis was not due to an up-regulation of this enzyme expression.

3. Generation of cysteine in the microenvironment by bone marrow stromal cells is critical to promote GSH synthesis and survival of CLL cells.

To maintain cellular GSH homeostasis and redox balance, the availability of the rate-limiting substrate cysteine is critical for GSH synthesis. Cysteine is a conditionally essential amino acid which can be synthesized from methionine only in certain tissues such as liver via the transsulfuration pathway [77], but many tissues including lymphoid cells have little capacity to synthesize cysteine due to a defect in transsulfuration [78]. Thus, their main source of cysteine is the uptake of extracellular cysteine or cystine through specific transporters [81-83]. Cysteine is transported by the ubiquitously expressed

ASC transporter (Na^+-dependent) as well as the Na^+-independent transporters [82], while cystine is mainly transported by Xc- and can be rapidly reduced to cysteine for GSH synthesis once inside the cells [85-88]. In addition, cells can also re-use cysteine from GSH through the γ-glutamyl cycle catalyzed by γ-glutamyl transpeptidase (GGT). However, CLL cells are known to have low GGT activity [132], and may not be able to effectively utilize extracellular GSH. Indeed, **Fig 18** showed that GSH under mini-molar range could not effectively protect CLL cells for survival. Due to their limited ability to take up cystine and to re-use GSH, CLL cells would mainly depend on the uptake of cysteine from extracellular environment for GSH synthesis. In plasma, cystine presents at 100-200 μM concentrations, while cysteine only exists at a much lower level in the range of 10-20 μM [88]. The low concentrations of cysteine in plasma reflect the dynamic balance between its constant generation from the tissue cells and oxidation extracellularly. Interestingly, 10 μM of cysteine could enhance CLL viability in the absence of drug treatment (**Fig 39**). This may explain why CLL cells have a relatively long survival time in the blood circulation. Higher concentrations of cysteine (20-200 μM) exhibited further protection of CLL cells and promoted drug resistance in a concentration-dependent manner (**Fig 39**). Thus, it is possible that when CLL cells are in a close proximity to the bone marrow stromal cells *in vivo*, the high local concentrations of cysteine near the stromal cells would provide strong protection for the leukemia cells leading to drug resistance.

Due to the chemical nature of cysteine, this thiol-containing compound was unstable in medium and could be detected only when the stromal culture medium was processed freshly and analyzed immediately (**Fig 33**). However, the cysteine signal lost after the

samples were stored and shipped for NMR analysis (**Fig 35**). Nevertheless, multiple lines of evidence strongly support the critical role of cysteine from the stromal cells to enhance GSH synthesis in CLL cells and promote their survival. (1) CLL cells cultured *in vitro* lost GSH rapidly and exhibited high spontaneous apoptosis in the absence of stromal cells; the low-molecular-weight (<3 kDa) portion of the stromal conditioned medium could restore GSH in CLL cells and increase their survival. (2) Addition of exogenous cysteine, cystine + 2-ME (to generate cysteine), or N-acetylcysteine to the CLL culture could significantly increase GSH synthesis in CLL cells and promote their viability without stromal cells. (3) Primary CLL cells showed an effective uptake of [^{14}C]-cysteine but not [^{14}C]-cystine, while the stromal cells could effectively take up both. In CLL cells cultured with [^{14}C]-cystine, the presence of stromal cells significantly enhanced the uptake of the radioactive material by CLL cells, suggesting that it was the stromal cells that converted [^{14}C]-cystine to [^{14}C]-cysteine for the CLL cells. (4) Cystine was required to maintain stromal viability and promote CLL GSH synthesis and drug resistance. (5) Stromal cells did not enhance the expression of xCT transporter in CLL cells, indicating that stromal cells did not promote cystine uptake by CLL cells via Xc-. This is consistent with the conclusion that stromal cells convert cystine to cysteine for CLL cells.

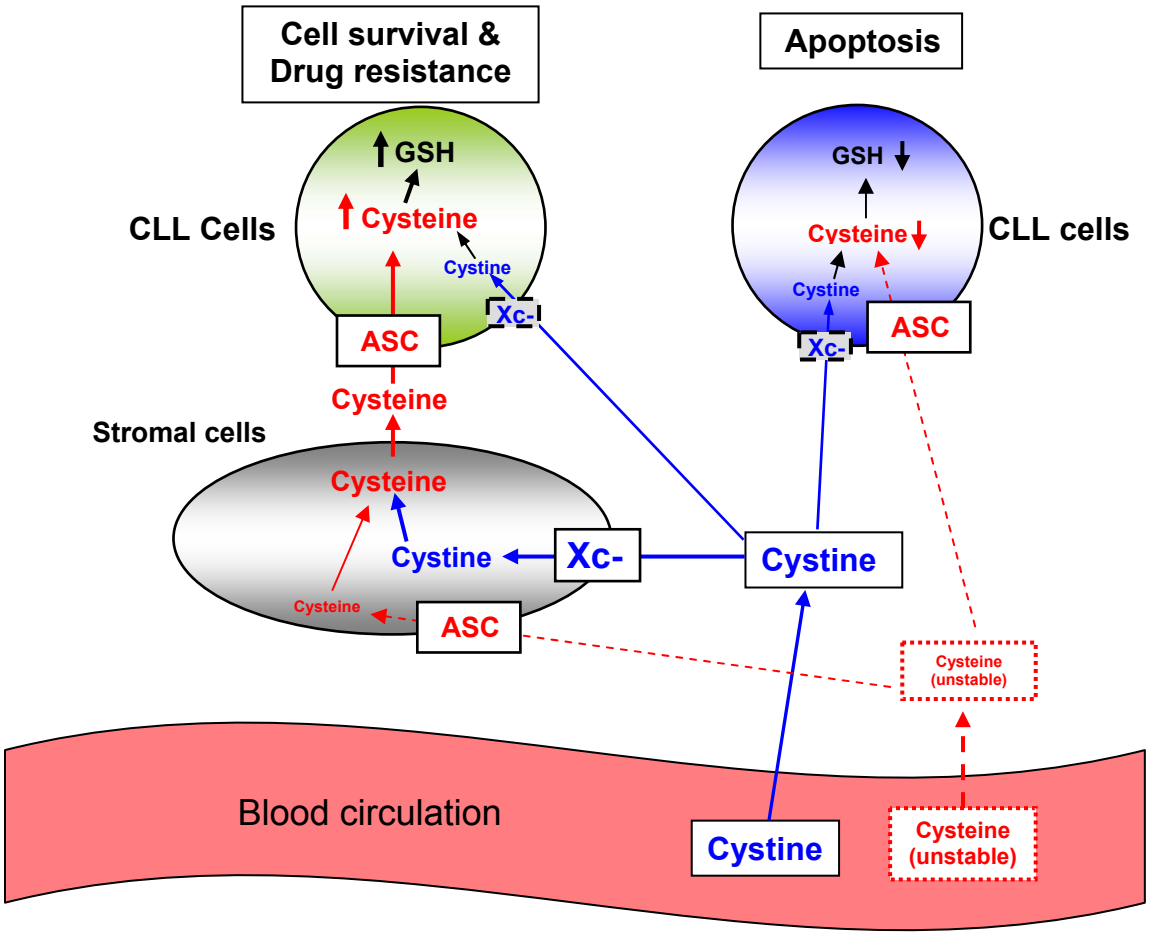
4. Biochemical pathway between bone marrow stromal cells and CLL cells.

While the role of GSH in promoting cancer cell survival and drug resistance has long been recognized [52, 63, 95, 133], the ability of bone marrow stromal cells to enhance GSH synthesis in CLL cells by providing cysteine as a critical substrate represents a previously unrecognized metabolic communication between the stromal cells and the leukemia cells. My study revealed a novel biochemical mechanism by which the bone

marrow stromal cells upregulate a major antioxidant system in CLL cells to maintain redox balance, and thus promote the leukemia cell survival. As illustrated in **Fig 63**, this biochemical pathway involves the uptake of stable cystine by the bone marrow stromal cells, the conversion of cystine to cysteine and its release to the microenvironment, and the uptake of cysteine by CLL cells for GSH synthesis to promote cell viability and drug resistance. Several important factors underscore the critical need for this biochemical pathway to protect CLL cells. The high intrinsic oxidative stress in CLL cells renders them highly dependent on GSH to maintain redox balance [52], but they have limited ability in the uptake of cystine as the GSH precursor due to the low expression of cystine transporter Xc-. Although cysteine can be transported by CLL cells, this compound is unstable in extracellular environment. The bone marrow stromal cells expressed a high level of Xc- transporter and were able to effectively take up cystine, which could then be converted to cysteine for use by CLL cells. It is possible that other tissue cells *in vivo* with high Xc- expression might also take up cystine and convert it to cysteine for GSH synthesis in CLL cells, and thus might protect the leukemia cells in a similar fashion as bone marrow stromal cells.

Interestingly, it has been observed that normal lymphocytes have low cystine uptake capability [134] and that macrophages can release cysteine to support the growth of lymphocytes [135]. However, the underlying mechanism remains unclear. My finding that CLL cells express low Xc- transporter provides an important molecular explanation for the low cystine uptake in CLL cells, and suggests that the leukemia cells and normal lymphocytes may share certain biological properties that are intrinsic to the lymphoid lineage.

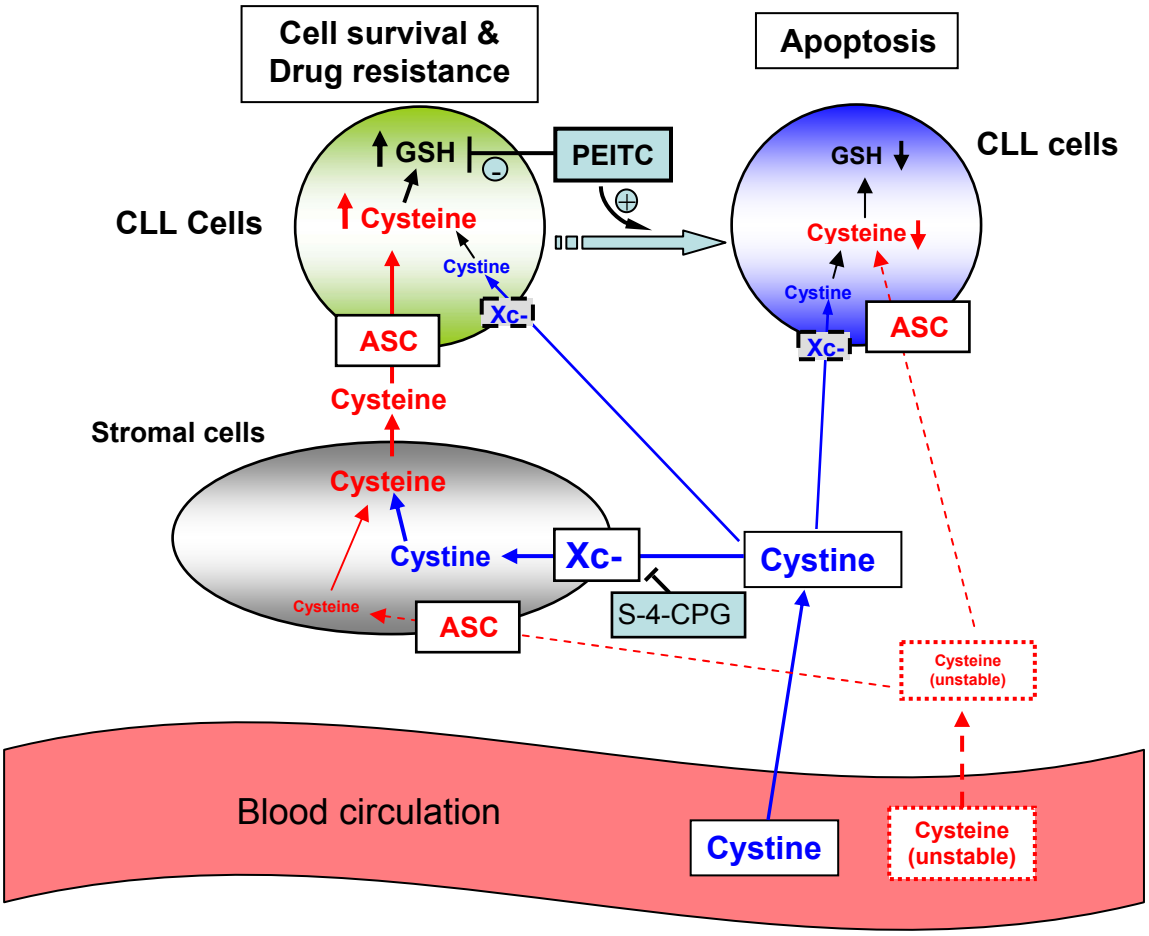
Figure 63. Proposed model for biochemical mechanism by which stromal cells enhance GSH synthesis in CLL cells and promote their survival, and strategies to overcome stromal-induced drug resistance. CLL cells express low level of cystine transporter Xc- and have limited ability to use the extracellular cystine for GSH synthesis. Although cysteine can be transported by CLL cells, this compound is unstable in extracellular environment. The bone marrow stromal cells expressed a high level of Xc- transporter, effectively take up cystine, and convert it to cysteine, which is released back to the extracellular environment for use by CLL cells to synthesize GSH and promote cell survival and drug resistance. Cysteine can be transported by both Na^+-dependent and Na^+-independent transporters. The ubiquitous Na^+-dependent transporter ASC is shown here.


Cell survival &
Drug resistance
Apoptosis
GSH
Cysteine
Cystine
Xc-
ASC
CLL Cells
GSH
Cysteine
Cystine
Xc-
ASC
CLL cells
Cysteine
Stromal cells
Cysteine
Cystine
Xc-
Cystine
Cysteine
ASC
Cysteine
(unstable)
Blood circulation
Cystine
Cysteine
(unstable)

5. Targeting the biochemical pathway to circumvent drug resistance.

Furthermore, I showed that the reliance of CLL cells on stromal cells for GSH synthesis could be exploited for therapeutic purpose by abolishing this protective mechanism to achieve effective killing of CLL cells in stromal environment. One prominent biochemical feature of CLL cells is their high ROS production [49, 52, 127]. Such intrinsic ROS stress renders the CLL cells highly dependent on GSH to maintain redox balance and thus critically rely on stromal cells to provide cysteine for GSH synthesis. As such, abolishing this stromal protective mechanism may represent a new therapeutic strategy targeting the Achilles heel of CLL cells. Indeed, my results from the proof-of-principle study using (S)-4-carboxyphenylglycine ((S)-4-CPG) to interrupt the cystine→cysteine→GSH flow or phenethyl isothiocyanate (PEITC) to abolish the GSH system suggest that such therapeutic approach can be effective in abrogating the stromal protection on CLL cells (**Fig 64**). Further evaluation of this biochemical intervention strategy in experimental systems and in clinical settings is important for the development of effective therapy to overcome drug resistance *in vivo*.

Figure 63. Targeting the biochemical pathway between stromal cells and CLL cells by (S)-4-CPG and PETIC. Inhibition of Xc- transporter by (S)-4-carboxyphenylglycine (S-4-CPG) or depletion of GSH by PEITC would abolish this biochemical protective mechanism, and increase the sensitivity of CLL cells to drug treatment in stromal microenvironment. Since CLL cells are under elevated intrinsic oxidative stress and more rely on GSH for survival, abrogation of the GSH mechanism would preferentially impact the leukemia cells and have high therapeutic selectivity (see text for detail).


Cell survival &
Drug resistance
Apoptosis
CLL Cells
GSH
PEITC
Cysteine
Cystine
Xc-
ASC
CLL cells
GSH
Cysteine
Cystine
Xc-
ASC
Cysteine
Stromal cells
Cysteine
Cystine
Xc-
Cystine
Cysteine
ASC
S-4-CPG
Cysteine
(unstable)
Blood circulation
Cystine
Cysteine
(unstable)

6. Summary and conclusions.

Tissue stromal cells interact with leukemia cells and profoundly affect their viability and drug response through yet undefined mechanisms. Here I show a biochemical mechanism by which bone marrow stromal cells modulate the redox status of chronic lymphocytic leukemia (CLL) cells and promote cellular survival and drug resistance. CLL cells from patients exhibit limited ability to transport cystine for glutathione (GSH) synthesis due to low expression of the Xc- transporter, while stromal cells effectively import cystine and convert it to cysteine, which is released into the microenvironment for uptake by CLL cells to enhance GSH synthesis. The elevated GSH protects leukemia cells from drug-induced cytotoxicity. Disabling this protective mechanism significantly sensitizes CLL cells to drug treatment in the stromal environment.

CLL is the most common adult leukemia in the western countries and is currently incurable due in part to drug resistance and the persistence of residual leukemia cells after chemotherapy leading to disease relapse. This study reveals a novel biochemical mechanism that mediates the interaction between the bone marrow stromal cells and leukemia cells through enhancing GSH synthesis to promote CLL cell survival and drug resistance. Importantly, I have identified pharmacological approaches that can effectively abolish this protective mechanism and sensitize CLL cells to standard drug treatment in the presence of stromal cells. The new mechanistic insights gained from this study provide a biochemical basis for developing new therapeutic strategy to overcome CLL drug resistance *in vivo*.

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VITA

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