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Investigating Apoptosis Pathway In Chronic Lymphocytic Leukemia: Stromal Influence And Therapeutic Activation

Viralkumar M. Patel

INVESTIGATING APOPTOSIS PATHWAY IN CHRONIC LYMPHOCYTIC LEUKEMIA:

STROMAL INFLUENCE AND THERAPEUTIC ACTIVATION

by

Viralkumar M. Patel

Approved:

_____
Varsha Gandhi, Ph.D.
Advisor

_____
Lynne Abruzzo, M.D., Ph.D.

_____
Hesham Amin, M.D.

_____
Jan Burger, M.D., Ph.D.

_____
George Calin, M.D., Ph.D.

Approved:

_____
Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston

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Viralkumar M. Patel, M.S.

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Dedication

To my loving parents and wonderful wife

Your encouragement, love and constant support have always kept me on continuing journey

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INVESTIGATING APOPTOSIS PATHWAY IN CHRONIC LYMPHOCYTIC LEUKEMIA:

STROMAL INFLUENCE AND THERAPEUTIC ACTIVATION

Viralkumar M. Patel, M.S.

Advisor: Varsha Gandhi, Ph.D.

Chronic lymphocytic leukemia (CLL) is a B-cell malignancy. High levels of Bcl-2 and IAP family proteins are responsible for apoptotic-resistance and accumulation of mature CLL lymphocytes in bone-marrow, lymph nodes and peripheral blood. Besides pro-survival proteins, supporting stromal cells as well as soluble factors in the microenvironment of bone-marrow and lymph nodes provide survival advantage to CLL leukemic cells.

Though the stromal – leukemia cell interactions has been studied extensively, in-depth-knowledge on the regulation of apoptotic pathway proteins in the context of microenvironment is still limited. To address this, the first part of our study focused on comprehensive analysis of 93 gene transcripts from seven families that are key regulators of apoptosis such as Bcl-2, IAPs, NF-kB, Caspase, TNF receptor superfamily (TNFRSF), Death Domain (DED) and Caspase Activation and Recruitment Domain (CARD) families using "real-time PCR apoptosis array-card". While shorter incubations with supporting stromal cells induced significant changes in 22/93 transcripts including Bcl-2 (6/22), TNFRSF (5/22), DED (5/22), Caspase (2/22) family members, longer incubations induced significant changes in 8/93 transcripts from NF-kB (4/8) and Bcl-2 (2/8) family members. At the protein level, decrease or stable expressions in pro-apoptotic proteins, but a significant increase in anti-apoptotic proteins was observed.

The second portion of this study involved therapeutic manipulation of executioner caspases in restoring apoptotic pathway that is frequently blocked by upstream anti-apoptotic Bcl-2 family members. A novel therapeutic approach that can potentially bypass the functional pro-survival proteins and directly activate terminal executioner procaspases to induce apoptosis was investigated. Executioner procaspase activities are blocked by zinc and zinc removal releases this inhibition. CLL primary cells express high levels of executioner procaspases-7 and -3 compared to normal lymphocytes providing a basis for therapeutic selectivity. Our studies with L14R8, a second generation PAC-1 analog [Procaspase activating compound-1 (PAC-1; Vanquish Oncology)] revealed that L14R8-induced cell death was specific to CLL cells and was independent of prognostic markers and microenvironmental factors. Mechanistically, L14R8 acted through direct activation of executioner procaspases -3 and -7 by removing labile Zinc ions. In summary, our investigations demonstrate cytotoxic actions of L14R8 and elucidate utility of this novel approach for combating CLL.

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List of Abbreviations

AKT	Protein Kinase B
ATM	Ataxia Telangiectasia Mutated
ANOVA	Analysis Of Variance
APAF1	Apoptotic Peptidase Activating Factor 1
APRIL	A Proliferation-Inducing Ligand
BAD	Bcl-2–Associeated Death Promoter
BAFF	B-Cell Activating Factor
BAK	Bcl2-Antagonist/Killer
BAX	BCL2-Associated X Protein
BCL-2	B-Cell Lymphoma 2
BCL2A1	Bcl2-Related Protein A1
BCL-XL	B-Cell Lymphoma-Extra Large
BCR	B-Cell Receptor
BH	Bcl-2 Homology
BID	BH3 Interacting Domain Death Agonist
BIK	BCL2-Interacting Killer
BIM	Bcl-2 Interacting Mediator Of Cell Death
BIR	Baculoviral IAP Repeat
BLYS	B Lymphocyte Stimulator
BMF	Bcl2 Modifying Factor
BNIP3	BCL2/Adenovirus E1B 19kda Interacting Protein 3
BOK	BCL2-Related Ovarian Killer

BSA	Bovine Serum Albumin
CARD	Caspase Recruitment Domain Family
CASP	Caspase
CD	Cluster Of Differentiation
CFLAR/CFLIP	Casp8 And Fadd-Like Apoptosis Regulator/Cellular Flice-Like Inhibitory Protein
CHOP	Cyclophosphamide, Doxorubicin, Vincristine, Prednisone ± Rituximab
CI	Combination Index
CIAP	Cellular Inhibitor Of Apoptosis
CLL	Chronic Lymphocytic Leukemia
CXCL	Chemokine (C-X-C Motif) Ligand
DAPK1	Death-Associated Protein Kinase 1
DED	Death Effector Domain
DIABLO/SMAC	Direct Iap-Binding Protein With Low Pi/Second Mitochondria-Derived Activator Of Caspase
DIOC6	3,3-Dihexyloxocarbocyanine Iodine
DKO	Double Knockout
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPM	Disintegration Per Minute
DR	Deat Hreceptor
ERK	Extracellular Signal-Regulated Kinases
ESRRBL1	Estrogen-Related Receptor Beta Like 1
FADD	Fas (TNFRSF6)-Associated Via Death Domain
FAS	Apoptosis-Mediating Surface Antigen

FBS	Fetal Bovine Serum
FFC	Forward Scattered
HIP1	Huntingtin Interacting Protein 1
HRK	Harakiri, BCL2 Interacting Protein
IAP	Inhibitor Of Apoptosis Protein
IC50	Half-Maximal Inhibitory Concentration
ICAM	Intercellular Adhesion Molecule
IG	Immunoglobulin
IGVH	Immunoglobulin Variable Heavy Cluster
IKB	Inhibitor Of Kappa Light Polypeptide Gene Enhancer In B-Cells
IKK	Inducible Ikappab Kinase
IL	Interleukin
JNK	Jun-Amino-Terminal Kinase
LRDD	Leucine-Rich Repeats And Death Domain
LTA	Lymphotoxin
MCL-1	Induced Myeloid Leukemia Cell Differentiation Protein
MEF	Mouse Embryonic Fibroblasts
MEK	Mitogen-Activated Protein Kinases
MOMP	Mitochondrial Outer Membrane Permeabilization
MRNA	Messenger Ribonucleic Acid
NAIP1	Neuronal Apoptosis Inhibitory Protein
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells
NIAP1	Nucleotide-Binding Oligomerization Domain, Leucine Rich Repeat And Bir Domain Containing 1

NLCS	Nurse Like Cells
NOXA	Phorbol-12-Myristate-13-Acetate-Induced Protein 1
PAC	Procaspase Activating Compound
PARP	Poly ADP Ribose Polymerase
PARP	Poly (ADP-Ribose) Polymerase
PBMCS	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline
PCA	Perchloric Acid
PEA15	Phosphoprotein Enriched In Astrocytes 15
PI	Propidium Iodide
PI3K	Phosphatidylinositide 3-Kinases
PKC	Protein Kinase C
PUMA	P53 Up-Regulated Modulator Of Apoptosis
Q-VD-OPH	(3S)-5-(2,6-Difluorophenoxy)-3-[[(2S)-3-Methyl-1-Oxo-2-[(2-Quinolinylcarbonyl)Amino]Butyl]Amino]-4-Oxo-Pentanoic Acid
REL	V-Rel Reticuloendotheliosis Viral Oncogene Homolog (Avian)
RIPA	Radio-Immunoprecipitation Assay
RNA	Ribonucleic Acid
RPM	Rotations Per Minute
RPMI	Roswell Park Memorial Institute Medium
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SEM S	Standard Error Of The Mean
SSC	Side Scattered
STS	Staurosporine

SYK	Spleen Tyrosine Kinase
TMRE	Tetramethylrhodamine, Ethyl Ester
TNF	Tumor Necrosis Factor
TNFRSF	Tumor Necrosis Factor Receptor Super Family
TP53	Tumor Protein P53
TRADD	Tumor Necrosis Factor Receptor Type 1 Associated Death Domain Protein
TRAF	TNF Receptor-Associated Factor
TRAIL	Tnf-Related Apoptosis Inducing Ligand
VCAM	Vascular Cell Adhesion Molecule
WBC	White Blood Cells
XIAP	X-Chromosome Binding IAP
ZAP70	Zeta-Chain (TCR) Associated Protein Kinase 70kda
Zn	Zinc
Z-VAD	N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) Fluoromethyl Ketone

CHAPTER 1: INTRODUCTION

1.1: Chronic lymphocytic leukemia (CLL)

1.1.1: Pathophysiology of CLL

Chronic lymphocytic leukemia (CLL), B-cell hematological malignancy is the most common adult leukemia in western countries [1]. North America and Europe are the major regions where CLL disease is predominant compared to other parts of the world. In US population, rate of occurrence is 3.9 incidences per 10,000 individuals, with the rate of incidence double in men, of median age 72 years [2]. The disease is commonly diagnosed in routine or other medical check-up. CLL is asymptomatic during diagnostic time in most of patients and the symptoms in advanced disease include enlargement of lymph node, bone marrow failure, and anemia [3] [4],[5], [6].

B malignant cells in CLL disease are small, mature lymphocytes [7] [8] and are replicationally quiescent in nature. Majority of CLL B-cells exist in G_0 or early G_1 phase of cell cycle and get accumulated in peripheral blood, lymph nodes, bone marrow and spleen [9], [10], [11]. Thus, CLL cells were thought to be slowly dividing but long-lived that resulting in their abnormal accumulation.   However, recent studies using heavy water (^2H_2O) labelling as a marker of cell proliferation in vivo demonstrated that CLL is a dynamic disease composed also of cells that proliferate and die, often at appreciable levels [12,13]. These malignant B-cells are identified with defective apoptosis process or Programmed Cell Death and thus inherit prolonged survival [11,14]. There are two streams of mechanisms that contribute to abnormal accumulation of CLL cells, 1) intrinsic defects of genetic or molecular origin [15], and 2) extrinsic signals from micro-environmental factors [16].

1.1.2: Intrinsic factors: Genetic aberrations in CLL

Defective apoptosis in CLL is contributed through many aberrations in chromosomes. Genetic alteration such as deletions, polysomy, less frequency, and translocation are considered major contributors in prognosis and management of disease. Trisomy 12 was the genetic abnormality first reported by Gahrton et al that is documented to be associated with poor treatment outcome [17], [18], [19], [20] and a less degree of Trisomy 3 has also been reported [21]. Chromosomal abnormalities in 13q14, 6q21, 11q22.3 and 11q23.1, 17p13 have been reported as common genetic aberrations in CLL [22], [23]. Deletion of specific genetic regions 17p13.1 and 11q22-23 associated with potential loss of TP53 and ATM genes, respectively were reported to associate with poor patient survival and treatment outcome, while 13q14.3 as the sole aberration is associated with favorable outcome [24], [25]. [26] [27]. Chromosomal translocation were reported for t(11;14)(q13;q32) and rarely for t(14;19)(q32;q13) [28], [29]. Additionally, genetic deletion at 13q14.3 is known to associate with loss of miR-15a and miR-16-1 [30]. This is the most common chromosomal lesion in this disease. Loss of miR-15a and miR-16-1 post-transcriptionally regulate the expression of anti-apoptotic proteins Bcl-2 and Mcl-1 [31] resulting in overexpression of these two anti-apoptotic proteins. This aberration directly relates genetic-dependent regulation of apoptotic pathway genes.

1.1.3: Intrinsic factors: Apoptotic imbalance in CLL cells

At the molecular level, transcriptional and translational abnormalities in apoptotic pathway proteins contribute to prolonged survival of CLL cells. For example, in the intrinsic pathway of cell death, apoptosis is often regulated by the Bcl-2 family members, which are considered the gate keepers of mitochondria. For a programmed cell death process, the expression levels of two sub-groups of Bcl-2 family proteins, anti-apoptotic and pro-apoptotic proteins is counter – balanced.

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However, in CLL malignant cells, due to high levels of anti-apoptotic proteins, the balance is frequently deregulated leading to resistance to apoptosis. High levels of Bcl-2 protein, low levels of Bax protein and concurrent elevation in the Bcl-2/bax ratio have been reported previously[32]. Polymorphic mutation of Bax identified in CLL, correlated with poor response to therapy[33]. The sensitivity of CLL B cells to apoptosis was consistently reported to be correlated with endogenous levels of Bcl-2 and Bax proteins [34]. In addition to Bcl-2 family proteins, IAP family members are also reported to be endogenously high in CLL [32], [35], [36] compared to control PBMCs or normal B cells and are associated with drug resistance and poor treatment outcome [37]. However, smac protein that is involved in inhibition of anti-apoptotic functions of IAPs, are found low in levels in CLL compared to PBMCs [38], [39].

1.1.4: Extrinsic factors: Contribution of microenvironment to CLL cell survival

Although CLL cells have intrinsic ability to live long in the body, they undergo spontaneous apoptosis in vitro.   This suggested that along with the innate anti-apoptotic factors, there should be some signals from extrinsic microenvironment that support the CLL cell survival.   A growing body of evidence suggests that CLL cells engage in complex cellular and molecular interactions with stromal cells and matrix.   The accessory cells involved in interaction with CLL cells are BMSC, NLC, DC, Vascular endothelial cells and so on.

BM stromal cells (BMSCs) were the first stromal cells characterized to support CLL cells [40]. CLL cells co-cultured on bone marrow stromal cells in vitro lived longer than cells cultured in media alone [40], [14]. Moreover, CLL cells co-cultured with murine fibroblasts promoted PI3K/NF-kB signaling and provided increase in anti-apoptotic Bcl-2 family member Bcl-X$_L$ and increased caspase inhibitors such as cFLIP and XIAP [41]. Both murine and human BMSC cells exhibited similar capacity to protect CLL cells from spontaneous apoptosis [42].   Bone marrow stromal cells modulate redox status of CLL cells and promote cellular survival and drug resistance through the mechanism of cysteine transport for glutathione synthesis[43].

Co-culture of CLL cells with vascular endothelial cells significantly enhanced the cell survival, an effect that was not seen with normal B cells.   Similar to BMSC, incubation of CLL cells with endothelial cells exhibited elevated levels of anti-apoptotic proteins Bcl-2, Mcl-1 and Bcl-XL in association with NF-kB activation [44]. Follicular dendritic cells rescue CLL cells from apoptosis through secretion of BAFF and IL-15, and expression of adhesion molecules like ICAM-1, VCAM-1, and CD44-induced mechanisms [45,46], [47], [48].

A proportion of CLL cells transforms into large round nurse-like cells (NLCs) on longer incubations during in vitro culturing [49]. NLCs are found in the spleen and secondary lymphoid

tissue of patients with CLL, and thus representing a model for lymph node microenvironment [50]. Importantly, gene profiling data on CLL cells derived from three compartments of CLL peripheral blood, bone marrow and lymph node suggested that the gene profile obtained for CLL-NLC co-cultures is similar to that obtained for lymph node samples.   NLCs express chemokines such as CXCL12, CXCL13, and other cell surface markers CD31 [51] and plexin B1, [52]. BAFF and APRIL, TNF family ligands derived from NLC, bind to TNF receptor family proteins such as B-cell maturation antigen (BCMA), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and BAFF receptor (BAFF-R) and activate canonical NF-κB pathway to protect CLL cells from apoptosis.

Accessory T-cells have also been a part of CLL microenvironment.   Proliferation centers contain a significant number of T cells expressing CD154 (CD40 ligand) [45], [53] that can bind to CD40 receptors present on CLL lymphocytes, and rescue them from apoptosis [54], [55], [56] [57], [58]. In tissue areas of pseudofollicles or proliferation centers, activated CD4$^+$ T cells co-localize with proliferating CD38$^+$ CLL cells, [59] indicating that T-cells promote the proliferation of CLL B-cells.   Recent findings show that in presence of IL-21 cytokine, CD40L signals derived from autologous T cells can induce antigen dependent [60] or antigen-independent [61] proliferation in CLL cells. In addition, T cells also demonstrated to increase survival through signaling pathways like NF-kB, PI3K/AKT, and MEK/ERK, pathways.   TNF-α, -γ, IL-13, and IL-2 are among many documented factors involved in T-cell induced survival to CLL cells [62,63], [64].

Another set of molecules that constitute microenvironment are adhesion molecules called integrins. They are heterodimeric glycoproteins having various α and β subunits and facilitate cell-cell or cell-matrix adhesion in the CLL-microenvironment milieu.   Several types of intergrins such as β_1 integrins (VLAs), $\alpha_4\beta_1$ integrin and VLA-4 (CD49d) are known to play an important role in the

interaction of normal and malignant hematopoietic cells and the bone marrow microenvironment. VLA-4 on CLL cells acts as a receptor for fibronectin and VCAM-1/CD106, and in co-operation with CXCR4 recptor promotes adhesion to BMSCs [65], [66]. While VLA-4 expression has prognostic impact on CLL cells [67], the expressions of ICAM-1 and L-selectin (CD62L) have been variable and broadly regulated by cytokines. Importantly, adhesion of CLL cells to fibronectin elevates Bcl-2 levels and increase Bcl2/Bax ratio [65]. Other important adhesion molecule in CLL includes CD44. Taken together, accessory cells, stromal cells, integrins, cytokines and chemokines together constitute the CLL microenvironment [68]. Figure 1 for illustrates of microenvironment factors contributing to CLL survival.

Figure 1: Illustration of microenvironmental factors involved in CLL survival
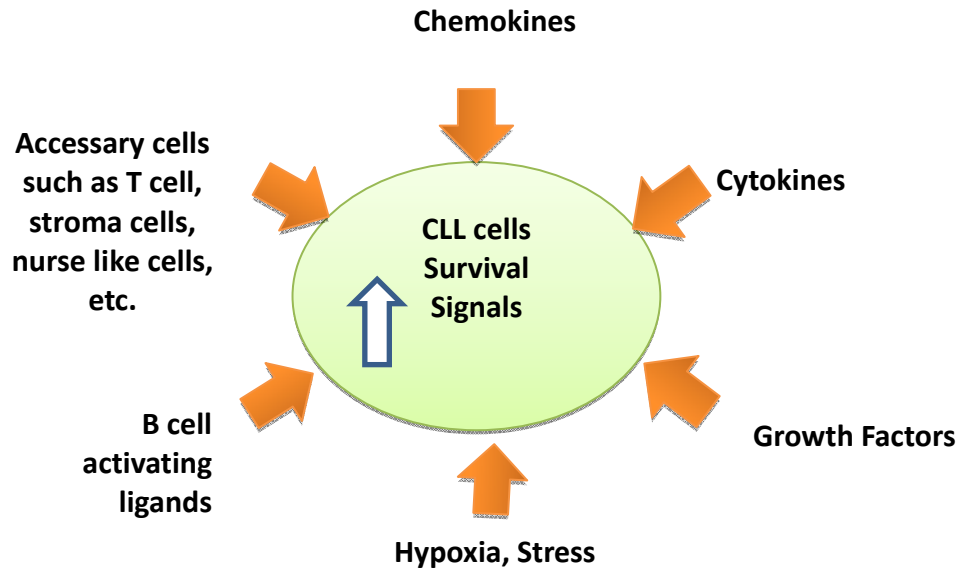
1.1.5: Bcl-2 family proteins and CLL microenvironment

CLL cells have defective cellular apoptotic machinery. Bcl-2 family anti-apoptotic proteins are major contributor for abnormal CLL survival. There are 26 Bcl-2 family members previously reported and 14 out of 26 Bcl-2 family members were of human origin. Human Bcl-2 families were categorized in to 3 sub families based on the functional Bcl-2 homology BH domains [69]. First subcategory, anti-apoptotic proteins included Bcl-2, Bcl-b, Bcl-x_L, Bfl-1, Bcl-w, and Mcl-1 proteins, function as inhibitors of apoptosis process. Critical functional importance has been evidenced for these six anti-apoptotic Bcl-2 family proteins. Bcl-2 protein documented for peripheral blood B cells population maintenance in adulthood [70], Bcl-x_L protein showed to be required for immature thymocytes survival [71]. Also, expression of Mcl-1 needed not only for hematopoietic stem cells survival but also for mature lymphocytes development. [72,73]. Besides this observation, over expression of Mcl-1 protein in transgenic mice induced B-cell lymphomas [74]. Knockdown of Bfl-1 transcript expression has been associated with induction of apoptosis in CLL cells [75]. Function of anti-apoptotic Bcl-2 family member Bcl-w, previously documented to be associated with sperm cells rather than lymphocytes [76].

Second subcategory is multi-domain pro-apoptotic proteins Bak, Bax, and Bok, which function as facilitator of the apoptotic cascade. Pro-apoptotic Bcl-2 family proteins function opposite to anti-apoptotic proteins and induce apoptosis. Bax and Bak are Bcl-2 family pro-apoptotic proteins and activate apoptosis thorough mitochondrial membrane permeabilization-induced cytochrome C release [77-79]. Programmed cell death process in lymphocytes requires expression of Bax protein [80]. Bak protein is involved in B-cell homeostasis, demonstrated in vivo. Also double knock down of Bak and Bak proteins in platelet have reduced apoptosis, and extended half-life [81].

Third subcategory is of BH3 only pro-apoptotic proteins which has 9 members. Bim, Bid which function as activator of BH3 only proteins and Bad, Bmf, Bik, Hrk, Bcl-rambo, Noxa, and Puma, which function as sensitizers of apoptosis [82]. BH3 only pro-apoptotic Bcl-2 family proteins function as activator or sensitizers of apoptosis. One member, Bid, function in myeloid cells population maintenance [83]; while another member Bim function in B cells program cell death, both in memory and plasma cells [84-87]. Also tumor suppressor role of Bad proteins in B cell is documented. [88].

Previously, Lagneaux et al. had demonstrated that CLL B-cells incubated with stromal cells increased survival compared to CLL cells in suspension and normal peripheral blood cells incubated with stromal cells increased apoptosis compared to normal cells in suspension. This indicates that malignant B-cells have altered signaling pathways compared to normal B-cells signaling pathway and obtain advantage from stroma cells or microenvironment [14]. It is now evident that CLL cells get partial prolong survival through interaction with stromal cells. CLL cells in bone marrow tissue microenvironment constantly interact and receive survival signals through pathways like B-cell receptor, Notch, Wnt signaling, CXCR4 receptor activation, etc. and activate transcription, translation or post translational modification of downstream anti-apoptotic proteins [89,90], [91]. Stoma-induced signaling in CLL increases expressions of IAPs such as cIAP1, cIAP2, Xiap [92], and Bcl-2 family members such as Mcl-1, Bcl-x_L, and Bfl-1 proteins. Studies also demonstrated that stroma-induced Mcl-1, Bcl-x_L and Bcl-2 proteins not only played important role in prolonged survival of CLL cells but also in resistance to spontaneous apoptosis and drug-induced cell death [93], [37,94,95]. Similarly, CLL patient lymphocytes co-cultured with lymph node microenvironment cell lines, example nurse-like cells, induced Bcl-x_L and Bfl-1 expression, which are other pro-survival factors playing role in CLL survival [11–13]. Another accessory cell type in microenvironment, follicular dendritic cells documented for Mcl-1 dependent extended survival of CLL cells and also

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rescued them from spontaneous apoptosis [50]. Involvement of other apoptotic family members in

CLL under diverse microenvironments described in next section of this chapter, however, there is

scanty data available for involvement of these apoptotic pathway members in CLL under stromal

microenvironment.

1.1.6: Microenvironment dependent regulation of apoptotic pathway proteins

Apart from Bcl-2 family proteins detailed above, various members from apoptosis pathway families are crucial for induction of apoptosis in context to CLL microenvironment. For example, Bcl-2 family, IAPs family, NF-kB signaling family, TNF receptor superfamily (TNFRSF) family, B cells receptors (BCRs), Death domain containing proteins (DED) family, Caspase activation and recruitment domain containing proteins (CARD) family, and caspase family. Among these families, Bcl-2, IAPs and NF-kB signaling family provide anti-apoptotic signals, and CARD, DED and Caspase family provide pro-apoptotic signals. Bcl-2, IAPs and NF-kB family members provide anti-apoptotic signals and are reported to be induced in presence of diverse microenvironment. Microenvironment-induced changes of these families critically contribute to apoptotic resistance in CLL cells. As described in the previous section, microenvironment-modulated expression of Bcl-2 family proteins rendering CLL cells more resistant. However, microenvironment not only documented to induce anti-apoptotic proteins in CLL lymphocytes but also reported to decrease pro-apoptotic signaling of caspases, DED, and CARD family members. Murine fibroblasts co-cultured with CLL B cells through PI3K/NF-kB signaling not only increase anti-apoptotic proteins but also reduced activation of apoptotic caspase-3 protein [41]. IL-21 expression in CLL B cells decreased caspase-8 and caspase-3 activation [96] while these reports exist, overall, information on microenvironment influence on caspases, DED, and CARD family members in CLL cells is limited.

Understanding pathways that could be influenced by microenvironment and detailed functional roles of these pathways and protein families are critical (Figure 2). Studies have documented involvement of many members of these families in apoptotic resistance of CLL cells with contexts of microenvironment influence (Table 1). TNFRSF and BCRs are surface proteins through which CLL cells interact with external microenvironment and receive pro- and anti-

apoptotic signals. TNFRSF family and BCR family members, being important upstream players of apoptosis pathway, function vitally in CLL-microenvironment interactions.

B cell receptor (BCR) is a major axis that is involved in survival, proliferation, progression, differentiation, adhesion and migration of CLL cells. BCR is made up of immunoglobulin heterodimer complex of CD79a and CD79b proteins on surface. This complex contains tyrosine activation motifs which upon antigen stimulation, promotes phosphorylation of proximal kinases such as SYK, PKC, AKT. BCR activation could be through antigen dependent and independent mechanisms. Prognostic marker such as ZAP70 positivity and IGVH unmutation were associated with increased BCR signaling and CLL cell survival [97], [98], [99], [100].

Similar to BCRs, other important signaling surface receptors include TNFRSF family members. TNFRSF family members such as TNFRSF6 (Fas receptor, CD95), TNF-α receptor 1 (TNF-R1 or CD120a), TNFRSF25 (death receptor 3), TNFRSF10A (death receptors 4) and TNFRSF10B (death receptor 5) interacts with extrinsic ligands such as TRAIL and induce death signals. Among them, Fas receptor activation in CLL demonstrated to induce cell death [101]. Activation of these receptors, through interactions with cytoplasmic adaptor molecules such as TRADD and FADD, mediates downstream caspase-8 dependent apoptotic signaling [102]. CD40 activated cytokine IL-21 expression in CLL B cells, which reduce pro-apoptotic signaling in CLL patient lymphocytes through reduction of caspase-8 and caspase-3 activation [96]. CD40L (CD154) induced pro-apoptotic Fas receptors expression on CLL cells, however, strong NF-kB signaling induction mediated overall anti-apoptotic effects in CLL cells [103,104]. Of the same family, TNFRSF7 (CD27), function as regulator of B cell receptor activation, was at high serum concentration in CLL patients and associated with poor prognostic factors such as high WBC count, clinical stage, and β2-

microglobulin expression [105]. These results support the notion of microenvironment-induced

regulation of pro-apoptotic factors from TNFRSF family in CLL cells to provide survival advantage.

Another type of TNFRSF family members such as TNF- α receptor 2 (TNFR2, CD120b), and

TNFRSF5 (CD40) provide anti-apoptotic signals upon interaction with extrinsic cytokines such as

BAFF, APRIL, and CD40L (Figure 2). CD40L (CD154) induced anti-apoptotic effect through activation

of CD40 receptors and induced NF-kB signaling mediated overall anti-apoptotic effects in CLL cells

[103,104]. Activation of these receptors mediates anti-apoptotic downstream signaling (NF-κB,

PI3K/Akt, JNK, ERK) through interaction with adaptor molecules from IAPs, DED family, and CARD

family such as TRAF interacting motifs proteins, cIAP1, cIAP2, and RIPKs (Receptor-Interacting

Serine-Threonine Kinases) [106]. In CLL cells, extrinsic ligand, BAFF (TNFRSF13 ligand) induced

survival of CLL cells through NF-kB signaling and increased anti-apoptotic proteins [107]. BAFF and

APRIL produced from diverse microenvironment were involved in CLL B-cell survival and

differentiation [108], [109,110]. Studies have demonstrated that BAFF and APRIL protect CLL cells

from spontaneous and drug-induced apoptosis. In addition, soluble BAFF protein and autocrine

feedback mechanisms of BAFF were demonstrated to provide survival in CLL cells [111]. Activation

of CD40 (TNFRSF5) receptors in CLL also associated with resistance to fludarabine treatment in vitro

[104]. CD4+ T cells released CD154 (CD40 ligand) interacts with CLL cells expressing CD40 receptor

(TNFRSF5) and up-regulated anti-apoptotic Bcl-2 family proteins such as Bfl-1, Bcl-x_L, Mcl-1, and

IAPs family member survivin [112].

Collectively, this context-dependent elevation of anti-apoptotic proteins majorly Bcl-2

family and deregulation of pro-apoptotic proteins suggests that many of apoptosis pathway family

proteins *per se* play a major role in the microenvironment-mediated survival advantage. Together,

accessory cells along with soluble factors significantly interact with TNFRSF and BCR family

members and contribute to the sustained survival of CLL cells by affecting pathways including NF-kB

signaling family and many common downstream anti- and pro-apoptotic proteins from Bcl-2, IAPs, DED, CARD and Caspases in CLL [113]. The balance between anti-apoptotic proteins (Bcl-2 family anti-apoptotic proteins, IAPs) and pro- apoptotic proteins (caspase family members, Death domain and CARD domain family proteins), dictates the fate of a CLL cell. Many gene screening profiles have been conducted to provide information regarding importance of these families in CLL-microenvironment contexts.

Experiments on screening gene profile in CLL patient cells obtained from different tissue environments such as peripheral blood, bone marrow, spleen, and lymph node were conducted previously. Studies by Herishanu et al. showed that lymph node microenvironment induced up-regulation of more than 100 genes involved in BCR signaling along with NF-kB signaling [114]. Gricks et al. attempted to evaluate gene expression profiling of CLL cells under CD40 activation compared with normal B-cells and found differential regulation of cell cycle and apoptosis associated genes [115]. Mouse bone marrow stromal cells-induced gene expression profiling in CLL cells have been carried out by Edelmann et. al that signified importance of PI3K/NF-kB signaling pathway [116]. Various gene profile experiments carried out in CLL in presence of model systems that mimic microenvironment identified many members from families of Bcl-2, IAP, TNFR and caspase of apoptosis pathway. However, comprehensive analyses of these families of apoptosis pathway in CLL and the impact of microenvironment are lacking.

Despite the notion that Bcl-2 family proteins are the central regulators of apoptosis in general and stromal-mediated survival in particular, an in-depth knowledge of the role of individual Bcl-2 family members in the context of the microenvironment and associated sustained survival is still limited. Additionally, the effect of microenvironment on other apoptotic pathway family proteins has never been studied. To address this issue, using a real-time polymerase chain reaction (PCR) mRNA array analysis, we investigated the impact of the bone marrow stromal

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microenvironment on gene transcripts of 93 genes that constitute families (Bcl-2, IAPs, NF-kB,

TNFRSF, DED, CARD, and Caspase) of apoptotic pathway in CLL primary cells.

Table 1: List of some of known anti- and pro-apoptotic proteins role in CLL under impact of microenvironments

Anti-apoptotic proteins			
Name of family	**Protein Name**	**Microenvironment/ligand**	**Function/Role**
Bcl-2 family	Mcl-1	Bone marrow	CLL survival [37]
	Bcl-2	Bone marrow	CLL survival [37]
	Bcl-xL	Nurse-like cells	CLL survival [11–13]
	Bcl-w	NA	Not in CLL [76]
	Bfl-1	Lymph node	CLL survival [11–13]
TNFRSF family	TNFRSF (CD40 receptor)	T cells / CD40 ligand	CLL survival and proliferation [112]
IAP family	cIAP1	Bone marrow	Inhibits caspases [92]
	cIAP2	Bone marrow	Inhibits caspases [92]
	XIAP	Bone marrow	Inhibits caspases [92]
	Survivin	CD40 ligand	CLL survival [112]
Pro-apoptotic proteins			
Name of family	**Protein Name**	**Microenvironment/ligand**	**Function/Role**
Death Domain (DED) family	TRADD	NA	Adaptor molecules [102]
	FADD	NA	Adaptor molecules [102]
Caspase family	Caspase-3	IL-21	Apoptosis in CLL [96]
	Caspase-8	IL-21	Apoptosis in CLL[96]
TNFRSF family	TNFRSF6 (Fas receptor)	Fas ligand	Apoptosis in CLL [101]

Figure 2: Schematic of functional role of protein families in apoptotic pathway

Extrinsic Pathway

B cell receptor

Btk, Syk, Lyn

MAPK pathway

PI3K/Akt pathway

NF-kB pathway

PI3Kδ

CD40R, TNFR2, BAFFR, ...

FasR, TNFR1, DR3, DR4, DR5, ...

TNF Receptor Superfamily

RIPK, IAPs (Ciap1, Ciap2), ...

Death Domain Protein family

FADD, TRADD...

Intrinsic Pathway

Bcl-2 family
Anti- and Pro- Apoptosis proteins

Anti-apoptotic pathways

NF-kB family

Pro-apoptotic pathways

Smac

Cytochrome C

IAPs

BIR Domain Protein family

Apaf-1...

CARD Domain family

Caspase-9

Caspases family

Caspase--2, -8, -10

Caspase -3, -6, -7

Apoptosis

1.1.7: Hypothesis I

CLL cells have inherent defective apoptotic pathway which protects these replicationally quiescent lymphocytes. In addition, survival of CLL cells is contributed by endogenous expression of pro-survival proteins and deregulation of anti- and pro- apoptotic members through extrinsic signals of microenvironment (Figure 1 and 2). Based on this background, I hypothesized that enhanced survival of CLL cells co-cultured with stromal cells should be dependent upon modulation of several apoptotic pathway proteins. This modulation should be reflected on levels of transcripts and proteins. Though the existing reports illustrate the effect of diverse microenvironments on selective proteins in apoptotic pathway, a comprehensive analysis on all the members of these families in the context of microenvironment was never done.

1.1.8: My Approach

To test my hypothesis, I have comprehensively analyzed 93 pro- and anti- apoptotic transcripts that could be categorized into seven families i.e. Bcl-2 family, NF-kB family, TNFR super family, Death domain family, CARD domain family, caspase family and IAP family (Figure 13-18). The assay was done using microfluidic technology for screening target expressions at mRNA level in application with real time PCR array. Following aims are studied to test the hypothesis.

Aim 1.1: To evaluate effect of stroma on viability and global transcription in CLL cells

Aim 1.2: To identify stroma induced changes in the transcript levels of 93 proteins that are associated with survival and apoptosis in CLL cells

Aim 1.3: To analyze stromal-induced changes in Bcl-2 family mRNA and proteins in CLL cells

1.2: Strategy to bypass survival signals through therapeutic activation of terminal procaspases in CLL

1.2.1: CLL therapeutics: standard of care

CLL is still incurable and is a recurrent disease except allogeneic stem cell transplant action. Since the median age of this disease is 72 years, stem cell transplant is also very difficult. Chemotherapy is currently one of the therapeutic options to combat CLL. Though current chemotherapies provide prolong survival in CLL patient, the disease is recurrent. Chemotherapy causes major decrease in CLL cell load in peripheral blood but it is less effective in lymph node and bone marrow microenvironment, which are the sites of CLL cells proliferation. Current standard of care chemotherapy includes alkylating agents, purine nucleotide analogues, and monoclonal antibodies.

Chlorambucil, bendamustine, and cyclophosphamide are alkylating agents used in CLL patients since many years. Fludarabine, cladribine, and pentostatin have been used as purine analogues. Fludarabine and cladribine treated patient showed overall drug response with better longer progression free survival [117,118]. Combinations of both alkylating agents and purine nucleoside analogs have higher anti-leukemic activity and progression free survival in patients than single agent treatment. Activities of human monoclonal antibodies such as rituximab, ofatumumab (for CD20 dependent B cell activation), alemtuzumab (campath; for CD52 protein), have added benefit to CLL treatment in large randomized phase III clinical trial by improving progression free survival, overall response rates, and survival in CLL patients [119].

1.2.2: CLL therapeutics: targeting BCR pathway

As mentioned in the previous section, 1.1.6, BCR signaling pathway plays important role in CLL disease. BCR signaling kinases such as bruton's tyrosine kinase (Btk), spleen tyrosine kinase (Syk), and phosphatidylinositol 3-kinase-δ (PI3Kδ) amplify signals and induce survival in CLL cells through activation of MAPK, PI3K/AKT and NF-kB signaling pathways (Figure 2). These signaling events are amplified due to over-expressions or constitute activations of these kinases. Syk is overexpressed in CLL and induces survival in CLL cells [120]. Syk also phosphorylates Btk, which function as intermediary enzyme in BCR signaling and is essential in B cell survival and signaling [121]. PI3Kδ isoform is overexpressed in CLL patient B lymphocytes compared to normal B lymphocytes [122].

Using small molecule inhibitors, targeting Btk by ibrutinib [122]), Syk by BAY61-3606 [123], and R406 [124], and PI3kδ by CAL-101 [125], induced apoptosis in CLL cells pre-clinical *in vitro* assays. Many of these agents are under clinical trials. Ibrutinib (PCI-32765) is a selective inhibitor of Btk and is currently under phase II clinical trial for patients with relapsed or refractory CLL (ClinicalTrials.gov Identifier: NCT01589302). GS-1101 (CAL-101) is an oral inhibitor of PI3Kδ and had completed Phase I clinical trial in patients with relapsed or refractory CLL by Gilead Sciences. Also CAL-101 is under phase III clinical trial with ofatumumab (CD20 human antibody) combinations for previously treated CLL patients (ClinicalTrials.gov Identifier: NCT01659021). Another PI3K inhibitor, IPI-145, by Infinity Pharmaceuticals, is under phase Ib clinical trial for hematology malignancies (ClinicalTrials.gov Identifier: NCT01476657).

1.2.3: CLL therapeutics: targeting Bcl-2 and IAP proteins

CLL is a prototype disease where neoplastic B-cells evade apoptosis due to over-expression of Bcl-2 and IAPs family survival proteins [32], [35]. These reports underscore development of targeted therapies that specifically and selectively target these proteins.

Many Bcl-2 family targeted therapies were tested preclinically for CLL. Small molecule inhibitors for Bcl-2 family anti-apoptotic proteins (Bcl-2 antagonists/BH3 mimetics include gossypol [39], AT101 [37], Obatoclax [126], ABT737 [127], ABT199 [128]) and inhibitors for IAPs (smac mimetics [129]; LBW242 [130]; SM-164 [131]) became a rational approach to combat this disease. These preclinical investigations lead to clinical trials along with development of second generation analogues as novel CLL therapeutics. Some of known Bcl-2 antagonists under clinical trials are ABT-263 [132], AT-101 [133], Obatoclax [134] for CLL therapeutics [135] [Reviewed]. Few of smac mimetics i.e. TL32711 for ovarian cancer and AT-406 for solid tumors and lymphomas are in clinical trials too. Among these, ABT-263 and its newer version ABT-199 demonstrate utility of this approach in CLL. However, activity of all these agents depend on the levels of endogenous Bcl-2 anti-apoptotic family proteins and pro-apoptotic proteins, in addition, they all activate apoptosis through intrinsic pathway that involve the mitochondria and Bcl-2 family proteins the gate keepers of apoptosis. Therefore pathway that is independent of anti-apoptotic proteins is needed.

1.2.4: CLL therapeutics: targeting terminal procaspases

In the general pathway of apoptosis, either intrinsic or extrinsic, the final execution of apoptosis is through activation of terminal (executioner) procaspases, functionally important enzymes called proteases. Bcl-2 family anti-apoptotic proteins and IAPs block the activation of downstream caspases, and inhibit apoptosis. Corollary to this observation is the fact that Bcl-2 antagonists or Smac mimetics that inhibit these two protein families induced apoptosis or programmed cell death. Thus, the key contributors of apoptosis are caspases.

In humans there are 11 caspases, however, only seven are involved with apoptosis [136] [Reviewed]. Among these seven caspases four belong to initiator caspases (caspase-2, -8, -9, -10) and three to executioner (caspase -3, -6, -7) caspases. There are two streams of apoptosis pathways, i.e. initiator-caspase-9-mediated intrinsic-apoptosis pathway that heavily involves mitochondria and initiator-caspase-8-dependent extrinsic-apoptosis pathway that initiates from death receptor axis (Figure 2). Both apoptosis pathways activate common downstream executioner caspases- 3, 6, and 7 [136] [Reviewed]. These investigations demonstrate critical role of executioner caspases in cell death in general and CLL lymphocytes in particular.

Executioner caspases are present in cells as inactive dimers known as procaspases. Activation of procaspases to active caspases is a prerequisite for initiation of programmed cell death [137]. After activation, these caspases cleave cellular substrates that have a C-terminal aspartate residue. One key element that is responsible for maintaining executioner caspases in its inactive procaspase configuration is chelation by intracellular zinc. Removal of intracellular zinc from cell has been shown to induce apoptosis in epithelial cells. Many researchers demonstrated cyto-protective actions of exogenous zinc ion. This observation provided an impetus to create small molecules that can chelate zinc [138]. Procaspase activating compounds (PACs) can convert

inactive dimers of executioner procaspases to their active cleaved forms by removing labile Zinc

ions from inactive dimer states and remove Zinc-mediated inhibition [139] (Figure 3). These agents

directly activate executioner caspases and bypass upstream survival factors [138]. First generation

PAC included and identified PAC-1.

Figure 3: Prcaspase-3 activation - role of zinc and PAC-1 mediated activation
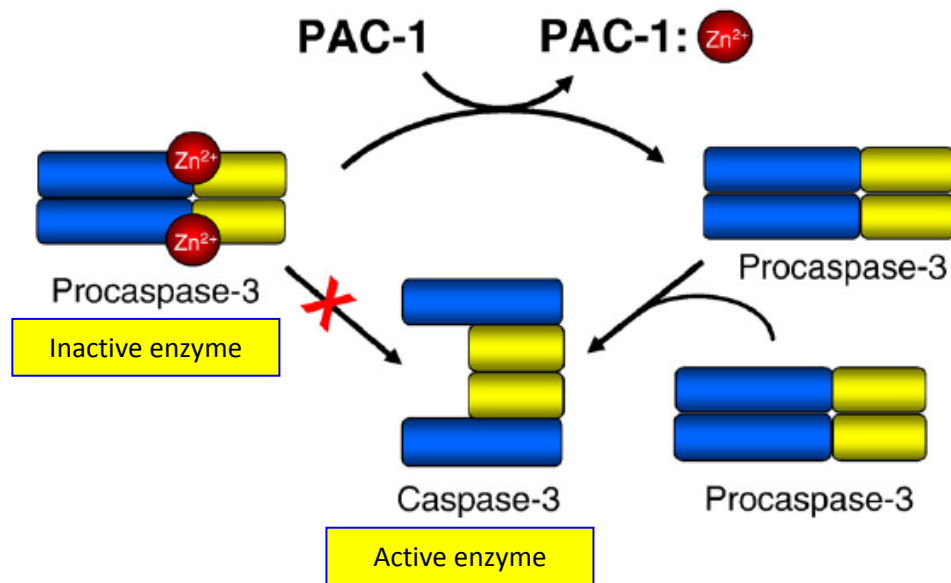
Modified from Publication Journal of Molecular Biology, Volume 388 /1, PAC-1 Activates Procaspase-3 in Vitro through Relief of Zinc-Mediated Inhibition, Pages No. 144-158, Copyright (2009), with permission from Elsevier."

1.2.5: Procaspase activating compounds

PAC-1 is a one of the procaspase activating compounds (PACs) that was identified in Paul

Hergenrother's laboratory at University of Illinois, Urbana, IL. These are now developed by

Vanquish Oncology, Champaign, IL. PAC-1 is an ortho-hydroxy N-acyl hydrazine that converts

inactive procaspase-3 to active caspase-3 both in in vitro biochemical assays and in whole cells.

Peterson et. al demonstrated detailed mechanism of action of PAC-1. PAC-1 converted executioner

procaspases to their active forms by removing labile Zinc ions. [139]. PAC-1 shown to inhibit tumor

growth of *in vitro*[138,140]'*[141]* and *in vivo[138,142]'* cancer models through zinc ion chelation of

executioner procaspases -3 and -7 [139].

Second generation PAC-1 combinatorial library analogues were designed and synthesized

to identify more potent and selective apoptosis-inducer in cancer cells [140]. Six library compounds

were selected that convert procaspase-3 to caspase-3 and induce cell death in U-937 cells. L14R8 is

the second generation PAC-1 analog which chemically contain benzyloxy and di-t-butyl functionality

(Figure 4). L14R8 has IC_{50} of 1.4 µM in a 3-day incubation assay in U937 cell line. Importantly,

incubation with 7.5 µM L14R8 resulted in almost complete cell death (97%) [140]. We have tested

utility of this novel compound in the second part of our study with CLL primary cells.

Figure 4: Structure of PAC-1 and its active and inactive analogues
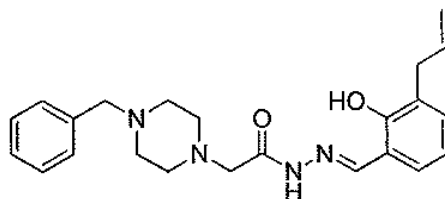
PAC-1
MW = 392.501 g/mol

PAC-1 structure
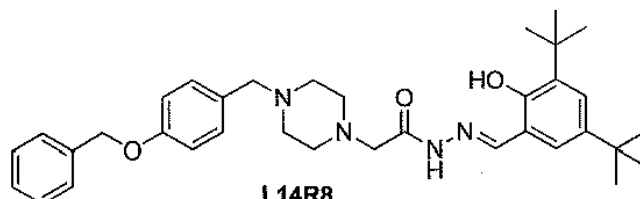
L14R8
MW = 570.775 g/mol

L14R8, a potent analogue of PAC-1
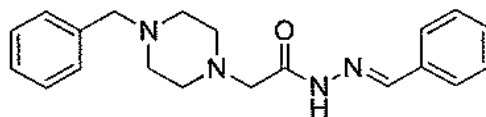
PAC-1a
MW = 336.437 g/mol

PAC-1a, inactive analogue of PAC-1

1.2.6: Hypothesis II

Based on survival advantage of CLL cells due to endogenous overexpression of anti-apoptotic proteins, their induction by microenvironment, role of caspases in CLL cell death, and actions of L14R8 in converting inactive procaspase-3 to caspase-3, I hypothesized that L14R8 will directly activate the functional ability of executioner caspases and should overcome the checkpoints of cell death to trigger apoptosis in CLL lymphocytes.

1.2.7: My Approach

To test the hypothesis, in the present project, I applied L14R8 in about 50 samples obtained from patients with CLL, identified mechanism of action, determined therapeutic index and tested combination approaches. Following aims addressed the hypothesis.

Aim 1.1: To investigate biological consequences of L14R8 treatment in CLL primary samples

Aim 1.2: To determine the mechanism by which L14R8 induces apoptosis in in CLL primary samples

Aim 1.3: To test mechanism-based combinations of L14R8 in CLL primary samples

CHAPTER 2: MATERIALS AND METHODS

2.1: Ligands, reagents and drugs

In the present study, following ligands, reagents and drugs were used: L14R8 [140] and PAC1a [139] (Vanquish Oncology), Z-Val-Ala-Asp-(OMe)-Fluoromethylketone (Z-VAD, MP Biomedicals, # 03FK00901), Q-VD-OPH (Q-VD, MP Biomedicals, # 03OPH10901), ABT-199 (Xcessbio, # M60075), Staurosporine (LC LABS, # S9300), Zinc Sulphate heptahydrate (ZnSO4.7H20, Sigma, # Z4750), Tetramethylrhodamine Ethyl Ester Perchlorate (TMRE, Life technologies, # T-669), IgM (MP Biomedicals, # 55055), Human Fas Ligand (Cell signaling, # 5452), Human CD40 Ligand (Cell signaling, # 3583), Human Interleukin-6 (Cell signaling, 8904), CD19 (Life Technologies, # MHCD1901), and CD3 stain (Life Technologies, # MHCD0301).

2.2: Cell lines

Jurkat wild type and caspases 8 (-/-) double knock out cell lines were generous gift from Dr. Joya Chandra laboratory, MD Anderson Cancer Center, Houston TX. Caspase 3 and -7 (Caspase-3/7) single allele (+/-) and double allele (-/-) knock out and wild type (WT) mouse embryo fibroblasts (MEFs) were generous gift from Dr. Besim Ogretmen laboratory, Medical University of South Carolina, Charleston, SC. For bone marrow stromal cells, human NKTert cell line was used. NKTert cell line was authenticated by STR DNA fingerprinting using the AmpFlSTR Identifiler kit (Applied Biosystems). Mouse embryo fibroblasts with Bax and Bak (Bax/Bak) wild-type (WT) or double knockout (DKO) were obtained from John C. Reed, Sanford-Burnham Institute, San Diego, CA.

2.3: Primary cell samples

We used three types of primary cells. CLL leukemic lymphocytes, normal peripheral blood mononuclear cells (PBMCs), and purified B-lymphocytes from healthy donors. First two types were

freshly isolated from peripheral blood samples obtained from either patients with CLL or healthy donors. All subjects signed written informed consent forms in accordance with the Declaration of Helsinki, and the laboratory protocols were approved by the institutional review board (IRB) at the University of Texas MD Anderson Cancer Center. Normal B-lymphocytes (Fresh/Untouched CD19+ B Cells) were purchased from AllCells, Alameda, CA (#PB010).

2.4: Isolation of lymphocytes

Peripheral blood from CLL patient or healthy donors was collected in heparinized green-top tubes and processed immediately for isolation of PBMCs. For this, collected blood was diluted in the ratio of 1:3 with cold PBS (2.7 mM KCl, 0.135 M NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 [pH 7.4]), and carefully layered on to high gravity Ficoll-Hypaque solution in 50 ml tube. The specific gravity of solution was 1.086 (Life Technologies, Grand Island, NY). The blood-PBS solution layered on Ficoll solution was centrifuged at 433g for 20 min. After centrifugation, tubes were carefully handled and interphase layer of white mononuclear cells was removed. Removed mononuclear cells were washed with PBS twice and centrifuged at 433g for 10 min to remove remnants of ficoll solution. Cells were resuspended with 10 ml media (RPMI 1640 + 10% Fetal Bovine Serum (FBS)) and were counted. Cells were cultured at $1X10^7$ cells per ml density and were incubated in 24 wells plate for further experiments. Cell number and mean volume of cells were measured using a Coulter counter with channelyzer (Coulter Electronics, Hialeah, FL).

2.5: Patient characteristics

Around 100 CLL patients sample were analyzed for the present study. Patient characteristics such as sex, age at sample collection, treatment status, IgVH mutation status, ZAP70 positivity, Rai stage, WBC count, β2M (β2 microglobulin) expression, and genetic aberrations (11Q (ATM), 17P (P53), 13Q, Trisomy 12 (T12), ND (Not determined)), of these samples were provided by clinical cytogenetics laboratory, Department of Hematopathology at M. D. Anderson Cancer Center (Table 2). IgVH mutation and ZAP70 positivity status were determined as previously described elsewhere [143]. Genetic aberrations were measured using FISH (Fluorescent in situ hybridization analysis) technique [144].

Table 2: List of patient samples used and patient characteristics

Patient No	Sex	Age at Sample	Treatment	IgVH Mutation status	ZAP70 positivity	Rai stage	WBC	β2M	Genetic aberrations
1	M	71	0	UNMUTATED	NEG	4	85.3	3.8	ND
2	F	59	0	UNMUTATED	POS	1	98.8	2.9	NEG
3	M	57	0	ND	ND	0	12.5	2.7	NEG
4	F	64	0	UNMUTATED	ND	2	31.1	2.7	NEG
5	M	77	1	92.9	ND	4	41.3	2.7	ND
6	M	67	0	UNMUTATED	NEG	2	214	4.5	11Q
7	M	82	2	MUTATED	POS	4	18	3.9	13Q
8	M	86	0	MUTATED	ND	4	81.7	5	ND
9	M	48	0	MUTATED	ND	1	10.6		NEG
10	M	56	0	ND	POS	1	109.9	2.6	11Q
11	F	61	0	MUTATED	NEG	1	138.7	3	NEG
12	M	68	0	MUTATED	NEG	1	11.4	2.2	ND
13	F	61	0	UNMUTATED	NEG	2	221.4		17P
14	F	68	0	ND	NEG	0	28.2	5.3	17P
15	M	70	0	MUTATED	ND	4	36.7	4.2	ND
16	F	62	1	UNMUTATED	ND	1	38	4.6	p53
17	M	35	0	ND	NEG	1	101.1	2.2	ND
18	M	57	0	MUTATED	ND	0	31.7	1.3	NEG
19	M	69	0	MUTATED	POS	0	26	2.2	NEG
20	F	77	0	MUTATED	NEG	2	159.1	7.6	13Q
21	M	64	0	MUTATED	ND	4	16.7	2.6	13Q
22	F	65	1	MUTATED	ND	1	93.5	ND	NEG
23	F	56	0	MUTATED	NEG	1	20.9	1.6	NEG
24	M	77	2	ND	NEG	0	64.5	ND	13Q
25	F	85	5	UNMUTATED	NEG	4	258.8	10.8	17P
26	M	60	5	UNMUTATED	ND	1	28.6	3.1	ND
27	F	79	0	MUTATED	ND	0	89.2	ND	ND
28	M	70	0	MUTATED	NEG	1	64.7	4.6	11Q
29	F	69	0	UNMUTATED	POS	1	81.4	3.3	11Q
30	M	71	0	ND	ND				
31	F	62	0	ND	POS	2	184.5	3.1	11Q
32	M	54	0	UNMUTATED	POS	2	113.8	3.1	17P
33	M	61	0	UNMUTATED	POS	2	49	4.1	11Q
34	F	77	0	ND	ND	1	92.9	6.1	ND

Patient No	Sex	Age at Sample	Treatment	IgVH Mutation status	ZAP70 status	Rai stage	WBC	β2M	Genetic aberrations
35	F	72	0	ND	ND	0	60.9	ND	ND
37	M	77	2	ND		1	75.4	ND	13Q
38	F	76	1	MUTATED	NEG				T12
39	F	66	0	MUTATED	ND	0	52.2	3	13Q
40	M	66	0	ND	ND	1	80.7	1.7	13Q
44	M	75.0	0	ND	POS	3	178.6	10.5	p53
45	M	73.0	0	UNMUTATED	POS	1	49.8	3.9	NEG
46	M	64.0	0	UNMUTATED	POS	2	222.3	5.4	p53
47	M	64.0	0	MUTATED	NEG	2	16.7	1.9	D13
48	M	69.0	0	MUTATED	NEG	1	67	2.2	NEG
49	M	47.0	2	ND	POS	1	45.7	2.7	D13
50	F	67.0	0	MUTATED	NEG	0	55.6	2	ND
51	M	43.0	0	MUTATED	POS	1	23.7	2.2	ND
52	M	81.0	2	MUTATED	NEG	2	39.7	5.6	ND
53	F	62.0	3	UNMUTATED	ND	4	119.7		11Q
54	M	56.0	1	UNMUTATED	POS	1	91.2	2.7	NEG
55	F	51.0	0	UNMUTATED	NEG	1	89.3	3.3	D13
56	M	49.0	0	MUTATED	NEG	3	111.4	3.1	ND
58	M	54.0	0	MUTATED	POS	2	129.5	5.1	D13
59	F	65.0	1	MUTATED	NEG	0	109	3.1	NEG
60	F	69.0	0	MUTATED	NEG	1	81.7	2	ND
61	F	63.0	0	UNMUTATED	NEG	3	132.3	9.3	p53
62	M	67.0	0	UNMUTATED	POS	2	104.1	4.4	ATM
63	F	61.0	0	MUTATED	NEG	0	169		ND
64	M	54.0	0	MUTATED	POS	2	123	5.1	D13
65	M	71.0	3+	UNMUTATED	ND	3	113.9		ND
66	F	87.0	0	MUTATED	NEG	0	28.9	2.9	ND
67	M	71.0	3+	UNMUTATED	ND	4	97.2		ND
68	F	73.0	3	UNMUTATED	POS	2	57.7	4.9	T12
69	M	51.0	0	ND	POS	1	98.2	2.6	ND
70	M	60.0	4	UNMUTATED	NEG	4	49	6	17P

Patient No	Sex	Age at Sample	Treatment	IgVH Mutation status	ZAP70 status	Rai stage	WBC	β2M	Genetic aberrations
71	F	59.0	0	UNMUTATED	POS	4	19.4	2.4	T12
72	M	54.0	0	UNMUTATED	POS	4	130.4	3.4	p53
73	F	51.0	0	UNMUTATED	NEG	4	68	3.7	D13
74	F	46.0	0	MUTATED	NEG	1	28.5	1.8	D13
75	M	52.0	0	MUTATED	NEG	0	28.5	1.7	ND
76	F	73.0	0	MUTATED	NEG	1	46.8	2.3	D13
78	M	68.0	0	ND	ND		33	3.7	D13
79	M	82.0	0	ND	NEG	4	104.1	5.1	T12
80	F	66.0	0	MUTATED	NEG	0	75.1	3.4	D13
81	M	61.0	0	UNMUTATED	POS	1	51.8	3.6	ATM
82	F	68.0	0	UNMUTATED	POS	1	122.8	3	p53
83	M	69.0	0	MUTATED	NEG	1	46.2	3.7	ATM
84	F	75.0	1	MUTATED	NEG	4	58.4	4.6	T12
86	M	61.0	0	ND	NEG	0	25.4	2.2	ND
87	M	41.0	0	MUTATED	NEG	2	102.1	2.4	ND
88	M	47.0	0	MUTATED	POS	1	71.2	1.9	D13
89	F	64.0	0	MUTATED	NEG	3	240.1	4	D13
90	M	47.0	0	MUTATED	POS	4	85.2	4	D13
91	F	69.0	0	MUTATED	NEG	1	107.6	2.1	nd
92	M	61.0	0	UNMUTATED	POS	1	33.2	2.4	p53
93	F	69.0	0	UNMUTATED	POS	1	51.5	3.2	p53
94	M	67.0	0	UNMUTATED	NEG	0	24.9	2.2	p53
94.1	M	77.0	2	ND	NEG	1	74		ND
95	F	68.0	0	ND	NEG	1	25.1	3.2	D13
96	F	55.0	0	UNMUTATED	POS	3	162.3	ND	p53
97	M	63.0	1	UNMUTATED	POS	2	48.4	3.1	D13
98	M	56.0	1	UNMUTATED	POS	2	184.9	2.6	D13
99	M	67.0	0		NEG	0	16.1	1.6	p53
100	M	56.0	0	MUTATED	NEG	1	80.1	1.3	ND
101	M	66.0	0	UNMUTATED	POS	4	114.5	3	p53
102	F	66.0	0	UNMUTATED	POS	1	52.7	2.7	NEG
103	F	67.0	0	MUTATED	NEG	0	214.6	2.6	D13
104	F	87.0	0	MUTATED	NEG	0	25.8	2.1	ND
105	F	59.0	0	UNMUTATED	POS	4	47.1	ND	T12
106	M	70.0	0	ND	POS	0	212	3.7	T12

2.6: Preparation of CLL-BMSC co-cultures

Isolated CLL lymphocytes (1 x 10^7 cells/mL) were cultured in suspension or co-cultured on stromal cells at the ratio of 1:100 (Figure 5). Human Bone Marrow Stromal Cells (BMSCs) used for experiments were NKTert cells (RIKEN cell bank, Tsukuba, Japan). To maintain the continuous cultures, NKTert cells were pre-incubated 5X10^4 cells per well in 24 wells plate with media (MEM alpha 1X (HyClone) + 12.5% human serum (Cellgro) + 12.5% FBS (SAFC Biosciences) + 100µM 2-mercaptoethanol (Sigma-Aldrich) and 1 µM hydrocortisone (Sigma-Aldrich)). After 24 hours, supernatant of NKTert cells were removed and 1 ml of 1X10^7 fresh isolated CLL cells in RPMI media were added to the confluent layers of NKTert cells. Cultures of CLL cells in suspension and on stromal co-cultures were kept at 37°C and floating CLL cells were carefully removed. To remove CLL cells adhered to NKTert cells, careful but vigorous washing of 1ml of PBS was applied and CLL cells were collected without disrupting NKTert layers. Floating CLL cells and PBS washings were centrifuged at 239g for 5 min and CLL pellets were stored at -80°C for further experimental analysis. Microscopic images of NKTert cells were taken as described below.

Figure 5: CLL lymphocytes and stromal cells co-culture model

CLL lymphocytes were isolated from blood by ficoll separation. 1 x 10^7 CLL cells per mL were co-cultured with confluent layers of NKTert human bone marrow stromal cells. The ratio of 100 CLL cells to 1 NKTert was maintained. Stromal cells were pre-incubated 16 to 24 hours in 24 wells plate before CLL coincubation.

CLL lymphocytes isolated by ficoll separation from blood

CLL lymphocytes

Human bone marrow stromal (NKTert) cells
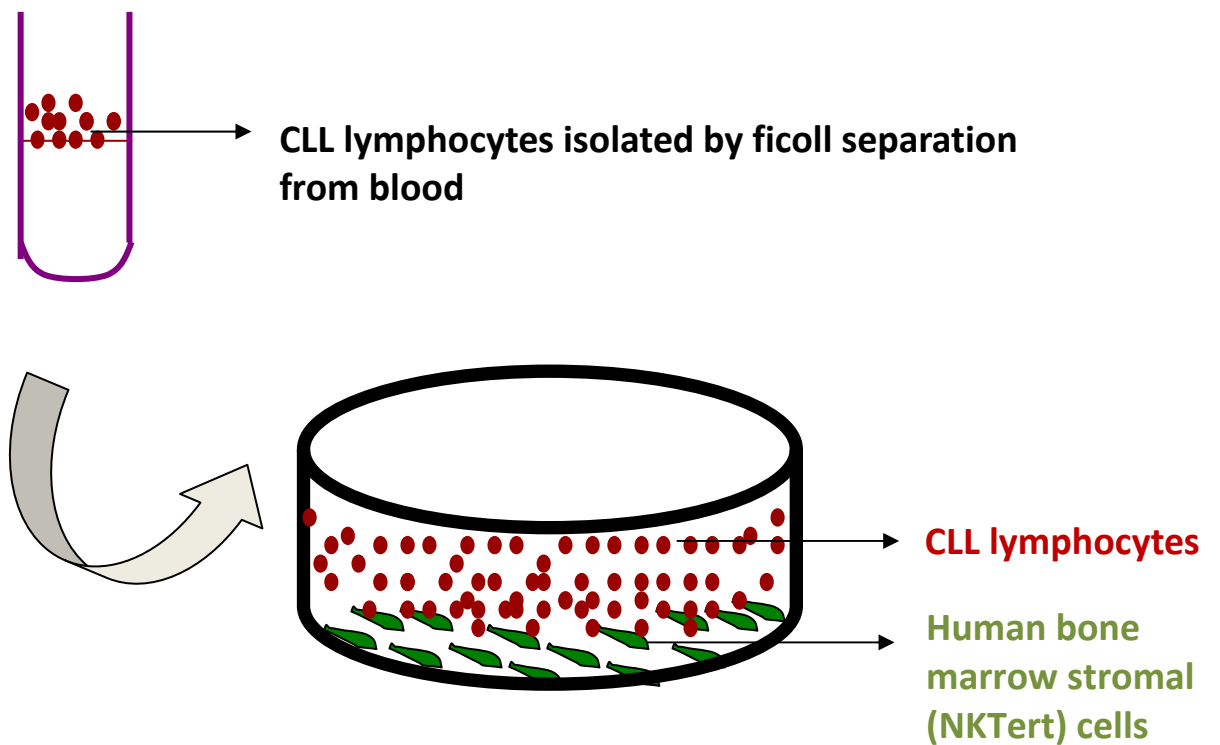
2.6.1: Quality control for optimal recovery of CLL cells from stromal co-cultures

Almost full recovery of CLL lymphocytes was needed for our experiment. To check this, using optical microscope (instrument series 1X70, Olympus) the images of CLL lymphocytes either in suspension or co-cultured with NKTert cells were taken (Figure 6, top panel). In both cases, wells showed healthy CLL cells in culture. At 24 hours after collection of CLL cells, images were taken again (Figure 6, lower panel). Both in suspension culture and in co-cultures almost complete recovery of CLL cells was achieved.

Figure 6: Microscopic images of CLL cells and stromal cells

Microscopic images were taken for CLL cells in suspension and on stroma at 24 hours coculturing in 24 wells plate (upper panel). Microscopic Images of stromal cells layer were taken after CLL cells collection and PBS washing (lower panel).
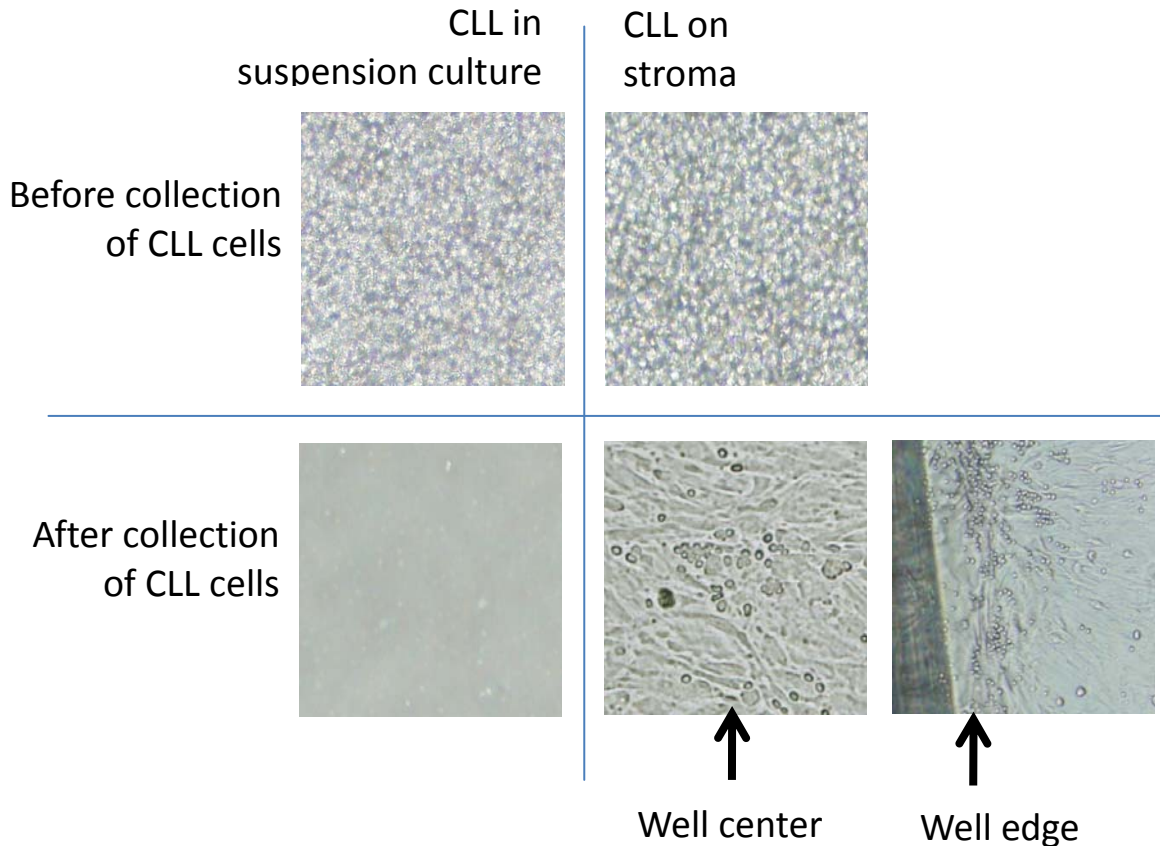
2.6: Treatment of isogenic MEFs and cell lines

WT, bax/bak (-/-), caspase 3/7 (+/-), and caspase 3/7 (-/-) MEFs were seeded at $5X10^4$/2 ml of RPMI media (+10% FBS) per well in 6-wells plate for 16 hours. Then, drugs and control treatments were added and incubated for 24 hours. Supernatant were collected and adherent cells were incubated in Accutase (Innovative cell technologies, AT-104) for 15 min. After washing with PBS, detached cells and supernatant were spun down for 5 min at 1500 RPM and preceded for Annexin V/PI cell viability staining as described below.

WT and caspase 8 (-/-) Jurkat cells were seeded at $1X10^5$/2 ml of RPMI media (+10% FBS + 1% glutamate for WT and + 15% FBS for Caspase 8 (-/-) jurkat cell lines) per well in 6-well plate for 16 hours. Then, drugs were added and incubated for 24 hours. Cells were collected and spun down for 5 min at 1500 RPM and preceded for Annexin V/PI cell viability staining as described below.

2.7: Cell viability assays

Two methods were used to test cell viability; these are Annexin V/PI and $DiOC_6$ staining.

2.7.1: Annexin V/PI staining method

Annexin V/PI staining method was carried out using Detection Kit I (PharMingen, San Diego, CA). 100 μL supernatant from suspension or co-cultures were collected and CLL cells (approximately $1X10^6$) were washed with PBS and collected as pellets. CLL cells pellet was resuspended in Annexin V/buffer solution (5 μL Annexin V–FITC in 1X annexin binding buffer; BD Bioscience) and incubated at room temperature in dark for 15 minutes. PI/buffer solution (10 μL PI Propidium Iodide (50 μg/mL) + 300 μL annexin binding buffer) solution was added just before analysis using FACScalibur cytometer (Becton-Dickinson). 10,000 cells per sample were counted to record Annexin V and PI positivity. Data was analyzed using Cell Quest (Becton-Dickinson) or FlowJo software (FlowJo, OR).

Data were expressed as % viability (Annexin V/PI negative), used for further analyses and comparisons.

2.7.2: DiOC$_6$ staining method

Another staining method used to measure cell death by means of mitochondrial transmembrane potential. Mitochondrial transmembrane potential was measured using 3,3-dihexyloxocarbocyanine iodine (DiOC6). Cell membrane permeability was measured using propidium iodide. 100 µL supernatant from suspension or co-cultures were collected and CLL cells (approximately 1X10^6) were washed with PBS and collected as pellets. CLL cell pellet was resuspended in DiOC$_6$ solution which was made up of 200 µL of 60 nM (Molecular Probes, Eugene, OR) and 10 µg/mL propidium iodide (50 µg/mL) in RPMI with 0.5% BSA, and incubated at 37°C in 15 minutes. Analysis was done within 30 min using FACScalibur cytometer (Becton-Dickinson). Flow cytometer filter setting for fluorescence was as following: FL-1 at 525 nm for DiOC6 and at FL-3 at 600 nm for PI. 10,000 cells per sample were counted to record DiOC$_6$ /PI positivity. Data was analyzed using Cell Quest (Becton-Dickinson) or FlowJo software (FlowJo, OR). Data were expressed as percentage of control.

2.8: Purity of CLL cell population

Purity of cell population was measured by two methods; first, by lymphocyte percentage and second, by CD19 staining. To determine purity of the B-cell population, we measured % lymphocytes in 12 samples. The % lymphocytes in these 12 samples ranged between 85-96% (median 92%) (Table 3).

Second, purity of CLL lymphocytes was measured using selective marker for CLL which is CD19 positivity (Figure 7). For this assay, 1X10^6 freshly isolated CLL cells in PBS were collected and 5

μL of antibody stain was added. The CLL cells were stained with CD19, a B cell marker and CD3 a T cell marker and the cells were incubated for 15 min and analyzed for CD19 and CD3 positivity using FACScalibur. 10,000 events per sample were collected. Data was analyzed using Cell Quest (Becton-Dickinson) or FlowJo software (FlowJo, OR).

Table 3: % lymphocytes present in CLL patient samples obtained for mRNA array analysis

The lymphocyte population in CLL patient samples indicated as % lymphocytes and was measured

for all 12 samples obtained for mRNA array analysis.

Patient No	% Lymphocyte
P27	93
P28	96
P29	96
P31	96
P32	92
P33	91
P34	89
P35	88
P37	90
P38	85
P39	86
P40	92

Figure 7: Purity of CLL B cell population

CLL cells from 12 patients were stained with CD19, a B cell marker and CD3, T-cell marker. % CD19

and % CD3 for representative samples are given (A). Patient (#40) graphs for flow-cytometry FFC

(forward scattered) and SSC (side scattered) analysis (B), CD19 and CD3 fluorescence staining (C),

and histogram of CD3 (D), and CD19 (E) staining in isolated CLL cell population from patient #40 are

presented.

A

Sample	CD19-PE	CD3-FITC
P27	ND	ND
P28	81.99	0.96
P29	81.99	0.96
P31	92.42	2.28
P32	ND	ND
P33	92.78	0.3
P34	93.8	0.01
P35	87.03	0.46
P37	90.63	2.62
P38	86.85	1.7
P39	88.46	3.85
P40	88.66	3.26

B
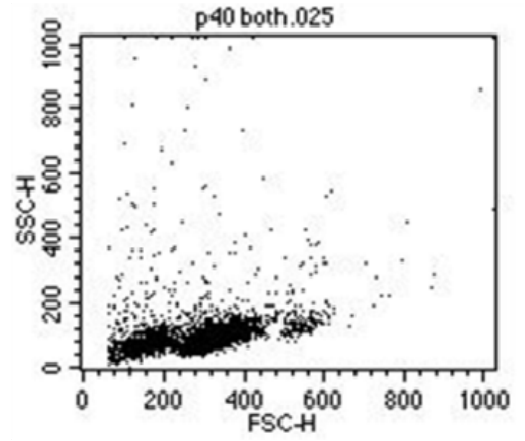
C
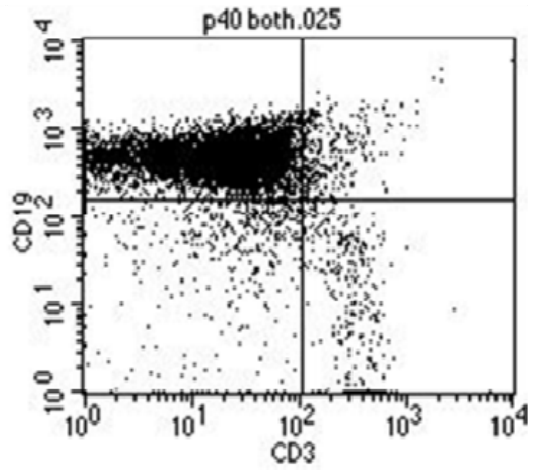
D
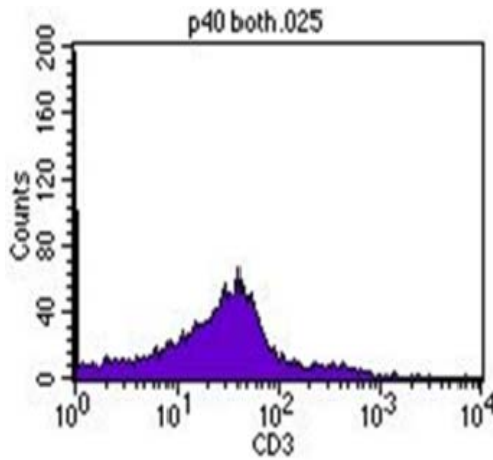
E
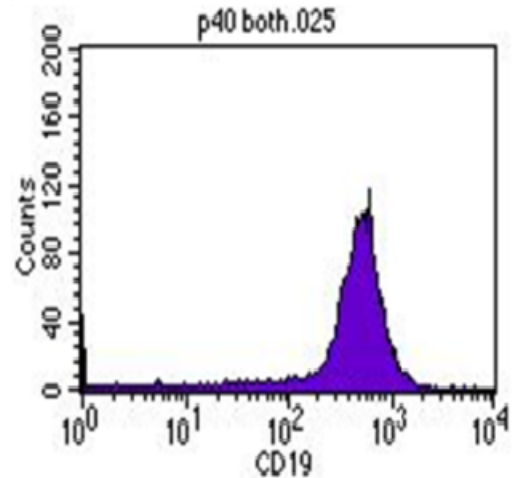
2.9: Mitochondrial outer membrane permeabilization (MOMP)

TMRE stain was used to measure mitochondrial outer membrane permeabilization. Briefly, 100-μL cell suspension was collected at the indicated time points, transferred to FACS tubes, washed with PBS and collected as pellets. Pellets were resuspended in 1000 μL of RPMI media and 1 μL of 25 mM TMRE were added and incubated for 30 min. Cells were washed and suspended in 300 μL of PBS. Histogram of TMRE stain was measured using FACScalibur (Becton Dickinson). Histograms were quantified as geometric mean using FlowJo software (FlowJo, OR).

2.10: Uridine Incorporation assay

Global RNA synthesis rate was measured in CLL patient lymphocytes using [3H] uridine incorporation assay. CLL lymphocytes were cultured in suspension or co-cultured on stroma for 12 hours, 1 day and 3 days. Afterwards, 10 mCi/mL [3H]uridine (Moravek Biochemicals, Brea, CA) per well was added for 30 min and incubated at 37^oC. Supernatant of cultures and co-cultures were collected and washed with 10 ml cold PBS followed by centrifugation at 239g for 5 min. CLL lymphocytes were washed with PBS again. Every step of procedure was carried out at 4^oC and on ice. After washing, CLL lymphocytes were pelleted, resuspended and vortexed in 500 μL water followed by addition of 500 μL of 0.8 N PCA and centrifugation. CLL cells were pelleted and resuspended in 1 ml of 0.4N PCA. CLL cells were again centrifuged, pelleted and resuspended in 1 ml water and 50 μL 10 N KOH. CLL cells were incubated at 37^oC overnight to dissolve RNA. Solution was transferred to scintillation vials and 7 ml of scintillation fluid was added. Radioactivity (DPM) was counted using liquid scintillation counter (instrument series - 1900CA, Packard)

2.11: Total RNA extraction and real time PCR assay

CLL lymphocytes were cultured in suspension or on stroma. Total RNA contents from CLL lymphocytes were extracted using RNeasy Mini Kit (Qiagen, 74106) and were quantitated using spectrophotometer (NanoDrop ND 1000, Thermo Fisher Scientific). Using Real time PCR transcripts expressions of BIM, Actin, 18S and Gapdh protein were measured. 5 μL of RNA solution containing 40 ng/mL for target genes and 5 ng/mL for control genes 18S, Actin, and Gapdh were prepared. The solution then added to one-step RT-PCR master mix solution (Cat # 4309169, Applied Biosystems) and 25 μL of reaction volume plated in 96 wells plate in triplicates. The reaction was run using 7900HT Fast Real-Time PCR detection system (Applied Biosystems). Primers and probes used for mRNA detection were as following: for BIM (Hs00708019_s1), for Actin (Hs99999903_m1), for Gapdh (Hs03929097_g1) and for 18S (Hs99999901_s1) (Applied Biosystems). BIM gene expression was normalized with endogenous controls (Actin, 18S and Gapdh), and average was taken as final measurement.

2.12: Real time PCR microfluidic card array analysis

In order to evaluate changes in transcripts of Bcl-2 family proteins in the presence of bone marrow stromal microenvironment, we used 'human apoptotic array micro fluidic card- Applied Biosystems' (4378701) for Real-time PCR. Briefly, CLL lymphocytes co-cultured with or without stromal cells were collected and processed for RNA extraction (Qiagen RNeasy Mini Kit, 74106). 2 μg of total RNA was converted to cDNA using High Capacity RNA-to-cDNA kit (Applied Biosystems, 4387406). 100 ng of cDNA and PCR master mixture (Applied Biosystems, 4304437) solution was introduced in each lane of micro fluidic array card. The array card was sealed and PCR reactions were carried out using 7900HT sequence detection system (Applied Biosystems). The data was analyzed using Applied Biosystems' softwares, RQ Manager and Data Assist. All mRNA expressions

were normalized to expression of three control genes β-actin, GAPDH and 18S. Used apoptosis array micro fluidic card contained 93 mRNA targets. These 93 mRNA targets included 18 mRNA targets of Bcl-2 family, 11 mRNA targets of caspase family, 8 mRNA targets of IAP family, 18 mRNA targets of CARD and DED family, 12 targets of TNFRSF family, 17 mRNA targets of NF-kB family, other, 2 mRNA targets of pseudo DED family (HIP1 and ESRRBL1), 8 mRNA targets of caspase and Bcl-2 family interacting proteins, and 3 control mRNAs (β-actin, GAPDH and 18S). Amplification curves for each individual transcript of Bcl-2, caspase and IAPs family in 12 patients were manually checked. All mRNA expressions were normalized to three control genes β-actin, GAPDH and 18S. Names of Bcl-2 family members, IAPs and Caspases analyzed using microfluidic card are presented as Table 5, 6, 8 and others are mentioned in the result section and in heat-maps (Figure 13-18) .

Real time PCR array card measured the transcript expressions using real time PCR reaction. However, to confirm that the results obtained from array card were not erroneous and possessed high quality accuracy, we had randomly selected BIM as target and compared results obtained from array card verses results obtained from individual real-time PCR, using same patient RNA lysate (Figure 8). BIM transcript expressions obtained from both the methods were significantly correlated.

Figure 8: Validation of Real-time PCR Array

To validate real time PCR array efficiency, BIM mRNA expressions measured through real time PCR array was compared with expression values obtained from individual RT-PCR run. Fold changes in BIM mRNA expression in CLL cells due to stromal cells coculturing for 12 hours, day 1 and day 3 incubations were measured. BIM mRNA expression detected through real time PCR array or through individual real time PCR using 96 wells plate were plotted for linear correlation.

$R^2=0.58$
P=0.0002
n=18
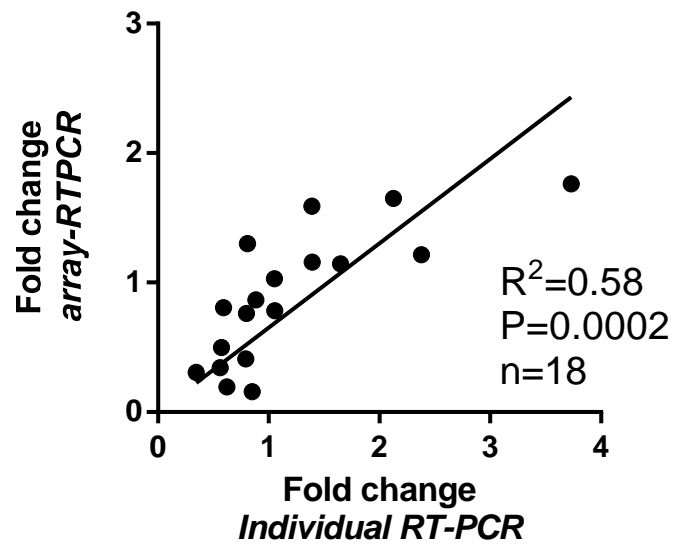
2.13: Immunoblot analysis

Protein lysate from CLL lymphocytes pellets was made using RIPA buffer (1 mL 10X RIPA lysis buffer (Millipore) + 9 ml water + one tablet of phosphatase (PhosSTOP, Roche) and one mini complete protease inhibitor (Roche). 80-120 µL of 1X RIPA buffer was added to CLL lymphocytes pellets and sonicated for 4 min with a 30 seconds interval at 4°C. Lysed cells were centrifuged for 10 min at 14,000 RPM speed and supernatant was carefully removed. All steps were performed on ice. Protein concentration of lysate was determined using a DC protein assay kit (Bio-Rad Laboratories). 5 µL of lysate was added to 96 well plate followed by addition of 25 µL of reagent A and reagent S (980 µL regent A + 20 µL reagent S) plus addition of 200 µL of reagent B. Plate reader (Powerwave XS, Bio-Tek) reading was taken at 750 nm, and using known standards, unknown concentration of protein lysates were determined by plotting linear graphs (Graph-pad prism software). Protein lysates preparation followed by SD-PAGE gel electrophoresis. 20-25 µg of protein lysates were boiled with sample buffer (XT Sample buffer dye, Cat # 161-0791 + XT Reducing agent, Cat # 161-0792, Bio-Rad Laboratories) at 97°C for 10 min. Prepared lysate-buffer solutions were loaded on 4% to 12% SDS-PAGE gels (Bio-Rad Laboratories) and were ran using 1X MOPS (Cat # 161-0788, Bio-Rad Laboratories) running buffer. Afterwards, proteins were transferred from gel to nitrocellulose membranes (GE Osmonics Labstore) using transferred buffer solution (700 ml millipore water + 200 ml methanol + 100 ml 10X buffer made up of 30gm Tris-base, 144 gm lysine and 100 ml water) at room temperature on ice for 45 min. Membranes were first blocked with odyssey blocking buffer solution (Cat # 927-40000, Odyssey) for 1 hour at room temperature and then blocked with primary antibodies for overnight at cold room or 2 hours at room temperature. Membranes were washed with Tween-PBS (0.5% Tween20 in PBS) for 15 min two times and blocked with infrared-labeled secondary antibody (1:5000 dilution; LI-COR Inc.) for 1 hour at room

temperature. Membranes were again washed for 15 min two times and scanned using LI-COR Odyssey Infrared Imager.

Primary antibodies used are as following: Rabbit polyclonal antibody to Bcl2-A1 (Epitomics, CA, 1639-1), Bcl-xL (Santa Cruz, SC634), Bad (Cell Signaling, MA, 9292), Puma (Cell Signaling, MA, 4976), Noxa (Santa Cruz, CA, SC30209), Bim (Santa Cruz, CA, SC11425), Mcl-1 (Santa Cruz, CA, SC819) and mouse monoclonal antibody to Bcl-w (Millipore, MA, AB1723), Bax (Santa Cruz, CA, SC20067), Bik (Santa Cruz, CA, SC365625), Bcl-2 (Santa Cruz, CA), and Gapdh (Sigma, St. Louis, MO). Also rabbit monoclonal antibody to Caspase-3 (Cell signaling, 9665), and polyclonal antibody to Caspase-9 (Cell Signaling, 9502), Caspase-6 (Cell signaling, 9762), Caspase-7 (Cell signaling, 9492), cIAP2 (epitomics, S2700), cIAP1 (abcam, ab2399), BIRC6 (abcam, ab19609), and NIAP (abcam, ab25968), and mouse monoclonal antibody to Poly (ADP-ribose) polymerase (Enzo life sciences BML-SA250), Smac (DIABLO) (BD Biosciences, 612246), XIAP (BD Biosciences, 610762), Caspase-8 (Cell Signaling, 9746), and Survivin (Santa cruze biotechnologies, SC-17779) were used. The Caspase-3 control cell extract (Cell signaling, 9663) was used to confirm procaspase-3 and active caspase-3 band.

2.14: Statistical analysis

Statistical analysis was performed using using the GraphPad Prism5 software (GraphPad Software, Inc. San Diego, CA). Linear correlations were used and mentioned as per analytical need. One sample t test, paired and unpaired student t-tests (two tailed), One-way ANNOVA and two-way ANNOVA were performed for statistical analyses and were mentioned at respective figure legends and/or text.

CHAPTER 3: RESULTS: Analysis of stroma-induced apoptotic targets in CLL

Hypothesis I

Enhanced survival of CLL cells co-cultured with stromal cells should be dependent upon modulation of several apoptotic pathway proteins. This modulation should be reflected on levels of transcripts and proteins. Though the existing reports illustrate the effect of diverse microenvironments on selective proteins in apoptotic pathway, a comprehensive analysis on all the members of these families in the context of microenvironment was never done.

This section of results (chapter 3) provides all data obtained for hypothesis I.

Aim 1.1: To evaluate effect of stroma on viability and global transcription in CLL cells

3.1: Stroma provided time-dependent survival advantage to CLL cells

CLL primary cells were co-cultured with human bone marrow stromal cells (NKTert cells) for six days and viability was measured using Annexin/PI staining (Figure 9A-9F) and $DiOC_6$ staining (Figure 9G). Compared to CLL cells in suspension, stroma provided cytoprotection and survival advantage to CLL cells. This protection was visible starting hours as 12 hours incubation of CLL cells (n=4) with stroma did not increase significant survival in CLL cells compared to suspension cells (p=0.222; data not shown). However, 24 hours co-culturing of CLL cells (n=25) with stromal cells increased significant survival of CLL cells (Figure 9A; p=0.0004). Longer incubations with stromal cells provided extended survival and cytoprotection to CLL cells. Co-culturing of stromal cells for 2 (Figure 9B; n=10; p=0.0107), 3 (Figure 9C; n=15; p<0.0001), 4 (Figure 9D; n=6; p=0.0246), 5 (Figure 9E; n=10; p=0.0038) and 6 days (Figure 9F; n=9; p=0.0232) provided survival to CLL cells and was statistically significantly different compared to suspension cultures. Also to confirm the results of

cytoprotection obtained from Annexin V/PI staining method, we had applied another staining technique of DiOC$_6$ (Figure 9G-L). Similar results were seen with this method as those obtained through Annexin V/PI staining method. When results were compared between both staining methods, there was strong and significant linear correlation (Pearson r = 0.94); (P<0.0001); (Figure 10; n=20).

3.2: Stroma induced time-dependent global RNA synthesis in CLL cells

CLL lymphocytes while replicationally quiescent are active in gene transcription. After measuring stroma-induced survival advantage, we carried out uridine incorporation experiment to measure stromal effect on RNA synthesis capacity by measuring collectively tRNA, rRNA and mRNA synthesis rate in CLL cells (Figure 11A-D). Similar to results with 12 hours viability, stroma did not cause significant change in global RNA synthesis rate in CLL cells (n=5; p=0.49). However, day 1 and day 3 co-cultured samples demonstrated significant increase in global RNA synthesis compared to CLL cells in suspension. The fold increase in radiolabeled uridine incorporation values for day 1 (n=13) and 3 days (n=8) were 1.7$\pm$0.5 (p=0.0019; Figure 11B) and 2.5$\pm$1.0 (p=0.029; Figure 11C) respectively. The collective data for all samples is presented in Fig 11D. In general, stroma-mediated cell survival was directly and linearly related to global RNA synthesis for the same samples (Day 1 and day 3; Figure 12; n=23; r=0.73; P<0.0001).

Figure 9: Effect of stromal cells on viability of CLL cells

CLL cells were co-cultured with NKTert stromal cells for different time periods from one day to six days. After incubation, CLL cells were collected, and measured for viability (A-F). Mean % viability of CLL lymphocytes incubated for 12 hours to six days both in suspension cultures and on stromal cell co-cultures were measured through Annexin V/PI staining method or through $DiOC_6$ staining method (G-L). Statistical analysis was performed using paired student t-test (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).
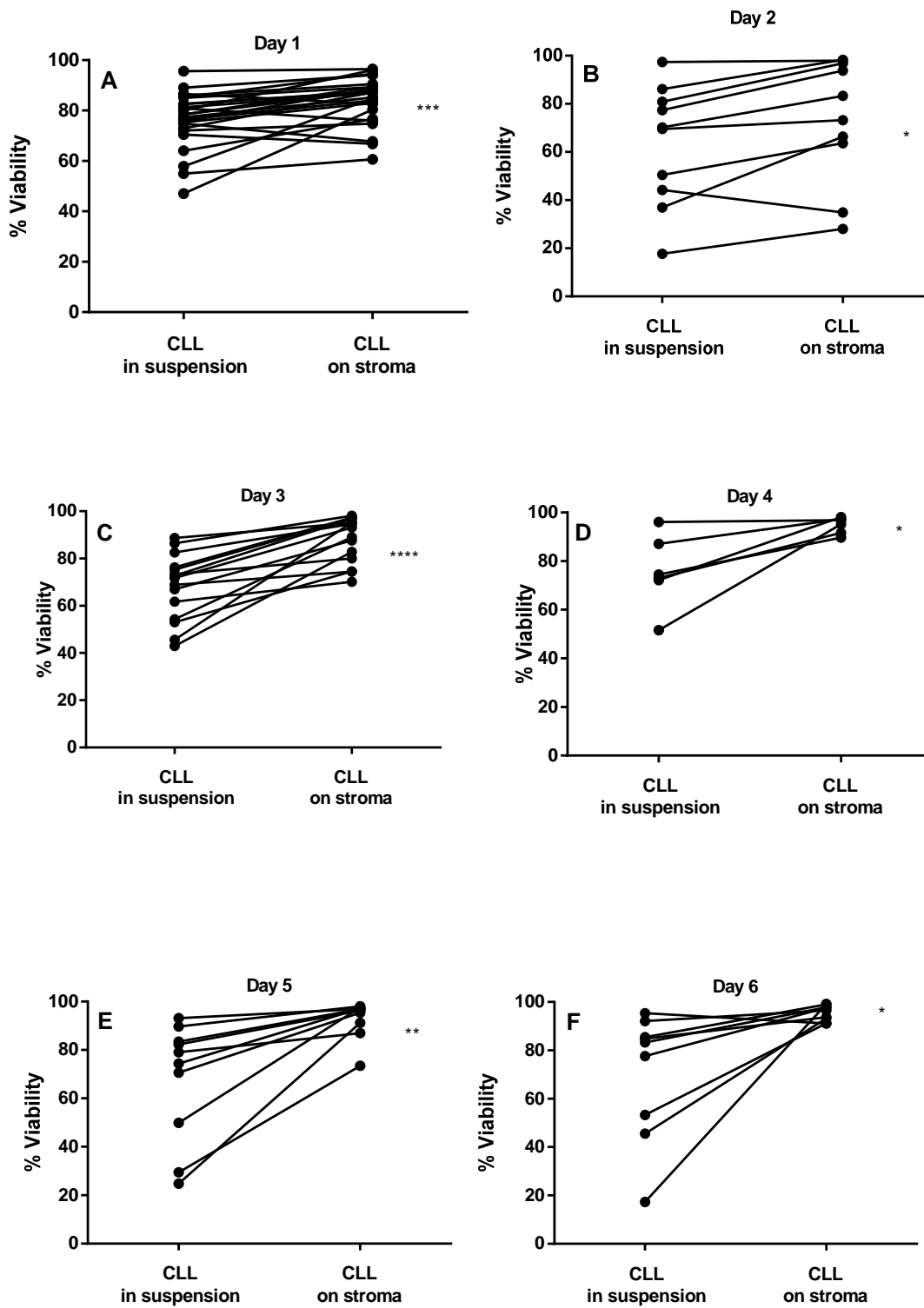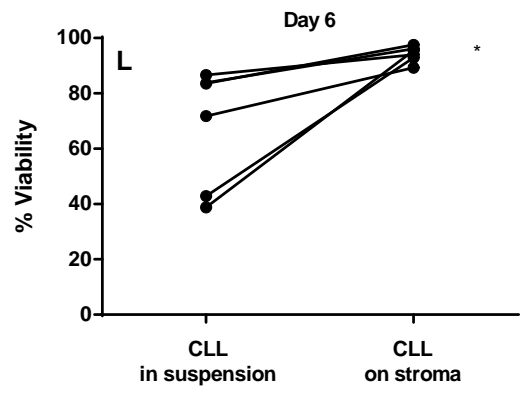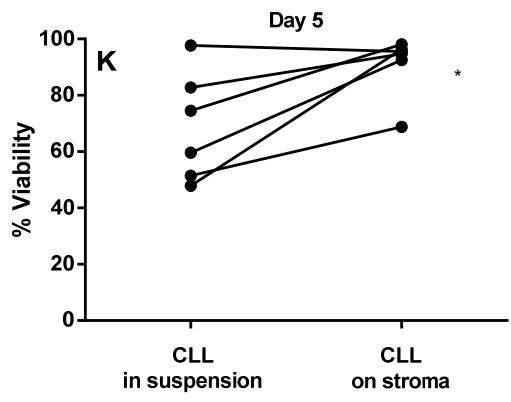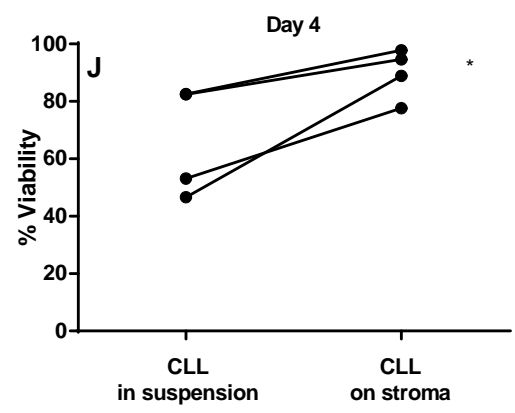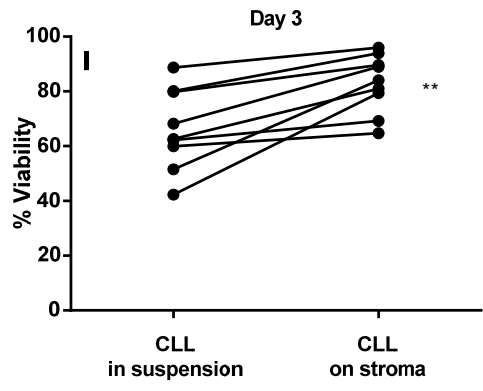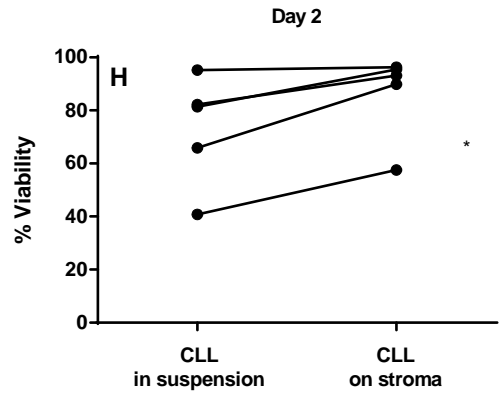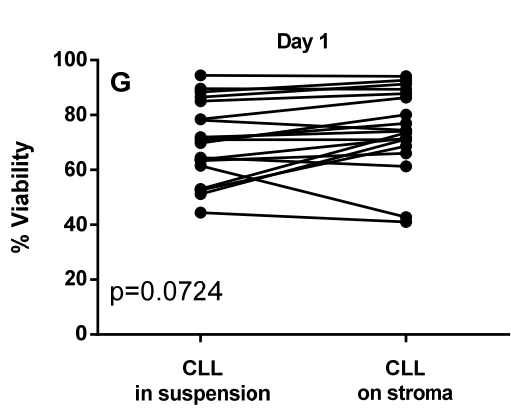
Figure 10: Comparison of Annexin V/PI and DiOC$_6$ staining method

% viability of CLL cells measured through Annexin V/PI and DiOC$_6$ staining method was compared. % viability of CLL cells in suspension was measured after 24 hours and correlation was measured through XY scatter plot for both the staining methods. Statistical analysis was performed using paired student t-test (****P<0.0001).
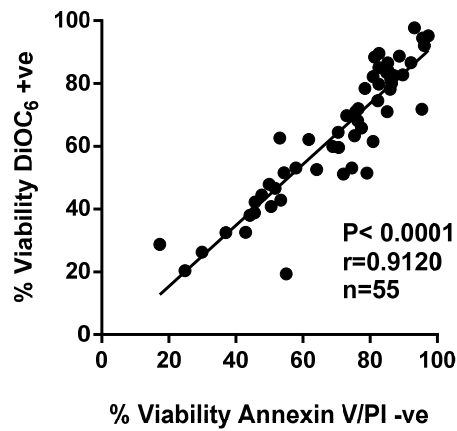
Figure 11: Effect of stromal cells on global RNA synthesis in CLL cells

CLL cells were co-cultured with NKTert cells and global RNA synthesis rate in CLL cells both in suspension and on stroma was measured through uridine incorporation assay after 12 hours (A), 1 day (B) and 3 days (C) of incubations. For each sample, assay was conducted in triplicate. Mean values of changes in global RNA synthesis in CLL cells due to stromal cells co-incubations were plotted (D). Statistical analysis was performed using paired student t-test (**P<0.01).
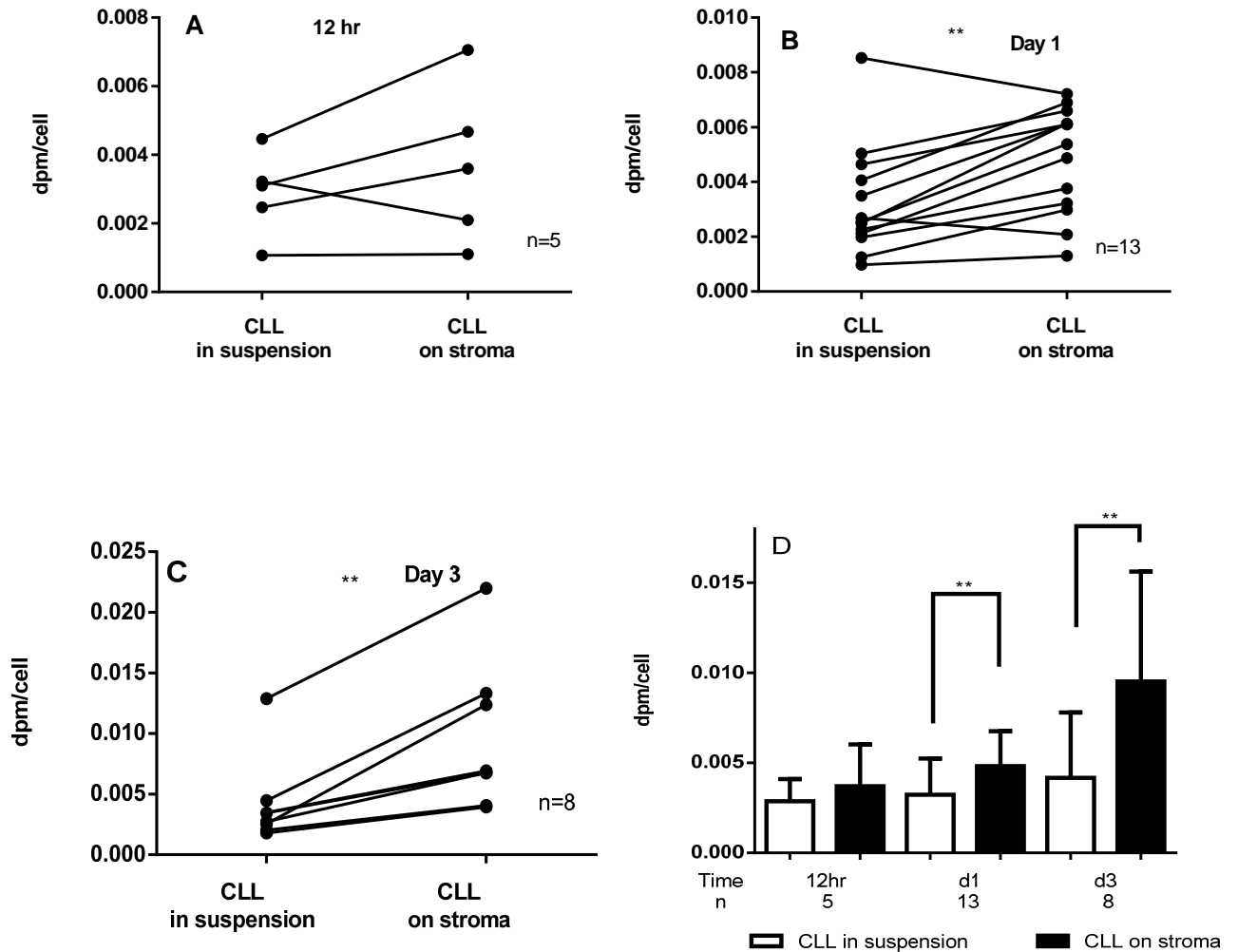
Figure 12: Correlation between survival and RNA synthesis for CLL samples

Correlation between % viability and % uridine incorporation measured in same CLL sample was plotted. % viability and rate of uridine incorporation in CLL cells co-cultured on stroma were measured at day 1 and day 3. Fold changes in viability and uridine incorporation in CLL cells due to stromal cells coculturing were plotted for linear correlation. Statistical analysis was performed using paired student t-test (****P<0.0001).
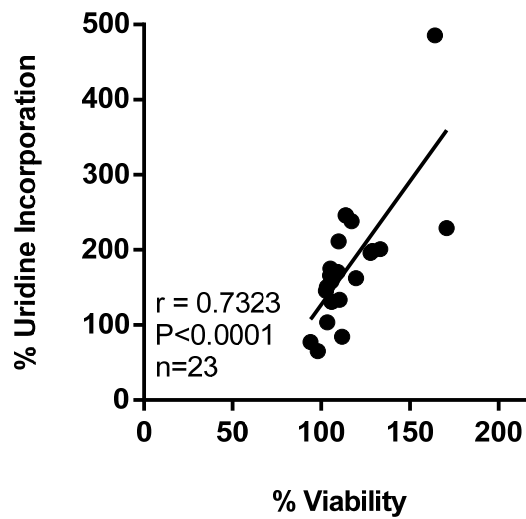
Aim 1.2: To identify stroma-induced changes in the transcript levels of 93 proteins
that are associated with survival and apoptosis in CLL cells

3.3: Stroma-induced changes in levels of transcripts of apoptotic targets in CLL cells

Given that stromal cells promoted viability and increased rate of global RNA synthesis, it

perhaps should induce changes in transcription of proteins involved in apoptosis pathways. Using

real-time PCR array (microfluidic technology), we have analyzed expression array of 93 genes in CLL

primary cells co- cultured with or without stromal cells (n=12). Assays were done after 12 hours

(n=4), 1 day (n=12) and 3 days (n=8) of culturing. These 93 apoptotic transcripts were categorized

into 7 families based on structural domains and functional relevance. Heat map of fold change in

transcripts of Bcl-2 family members (Figure 13), NF-Kappa beta family members (Figure 14), BIR

domain family members (Figure 15), CARD and DED domain family members (Figure 16), Caspase

family members (Figure 17), TNFR super family members (Figure 18) are presented.

Using cutoff of significant change at p=0.05, 22 and 8 transcripts hits were identified for day

1 and day 3, respectively (Table 4). Among top 22 hits recognized for day 1 samples, 6 hits were

from Bcl-2 family, 5 hits from TNFRSF family, and 5 hits were from Death Domain proteins family.

Interestingly, caspase-3 and caspase-6 were also among these. HIP1 and ESRRBL1 resemble pseudo

DED domain and previously reported to be involved in survival of hematology malignant cells, were

among top hits. Among top 8 hits from day 3 samples, 4 hits were from NF-kB family, and 2 hits

were from Bcl-2 family and DIABLO/SMAC transcripts.

Among the 18 Bcl-2 family members analyzed, 13 were detected in CLL cells. We studied

this family of proteins in detail because of their significant role in the mechanism of CLL cell survival

(Table 4, Figure 13, 20-23) and have described them later. There was no change in transcripts of

BNIP3 (NAIP3), and BNIP3L, the proteins that function by interacting with Bcl-2 family.

Another family analyzed was transcription factor NF-kB complex members and members which interact with NF-kB function (Figure 14). NF-kB subunit, Rel family members analyzed were NFKB1 (p50), NFKB2 (p52), REL (c-Rel), RELA, and RELB. Inhibitors of NF-kB such as IkB family members, NFKBIA (IKBA), NFKBIB (IKBB), NFKBIE (IKBE), NFKBIZ (IKBZ), TA-NFKBH (IKB Delta), and BCL3, which sequester inactive NF-kB, were also analyzed. Also factors which activate IkB such as CHUK (IKKalpha), TBK1, IKBKB, IKBKE, and IKBKG, were included. Among these members, stroma did not induce any significant change on day 1(except NFKBIA; Table 4); CLL cells co-cultured on stroma for longer time induced significant decrease in NF-kB transcription complex subunit, NFKB1 (p50; p=0.0338), and NFKB2 (p52; p=0.0568), and decrease in IkB family members such as NFKBIE (IKBE; p=0.0310), and NFKBIZ (IKBZ; p=0.0003) that inhibit NF-KB activation (Table 4).

Eight members of Inhibitors of Apoptosis Proteins (IAPs) family mRNAs were in this array card. These include BIRC1 (NAIP1), BIRC2 (cIAP1), BIRC3 (cIAP2), BIRC4 (XIAP), BIRC5 (Survivin), BIRC6 (Bruce), BIRC7 (Livin), and BIRC8 (ILP2) (Figure 15). Out of 8, BIRC7 (Livin) and BIRC8 (ILP2) transcript expressions were not detected in CLL cells (Figure 15; Table 8). Interestingly, transcript levels of inhibitors of IAPs, DIABLO/SMAC, decreased significantly in CLL cells after 3 days of co-culturing (median=0.72; p=0.0301; Table 4).

Death Domain containing proteins regulate inflammation and apoptosis process by interacting with proteins of caspases, NF-kappaB, and TNFRS families (Figure 16) [145], [146]. Members such as DAPK1, DEDD, DEDD2, FADD, LRDD, TRADD, RIPK2, and PEA15 are in Death Domain containing proteins family. Increase in adaptor molecules such DEDD, DEDD2, FADD, and TRADD was observed during shorter incubations (See Table 4).

Ten members of CARD domain containing proteins are APAF1, CARD15, CARD4, CARD6, CARD9, CRADD, ICEBERG, NALP1, RIPK1, PYCARD (Figure 16). Among them, ICEBERG was below

level of detection in all samples. Stroma induced significant increase in transcript expression of NALP1 (p=0.0012; Table 4), at shorter incubations which function as procaspase-9 activator in apoptosis induction, but did not change during longer incubations.

Twelve members of caspase family proteins were analyzed (Figure 17). Initiator caspases (CASP2, CASP8, CASP9, CASP10), executioner caspases (CASP3, CASP6, CASP7) and inflammatory caspases CASP1 and CASP4 were endogenously present while CASP5, and keratinocyte caspases (CASP14) was below of detection limit (Table 6). Two important factors of caspase-8 complex, CFLAR and CASP82 were present at detectable levels. CFLAR functions as inhibitor of Caspase-8 complex activation was increased significantly under stromal incubations on day 1 (p=0.0125). Moreover, except two members of caspase family, all of the transcripts were unchanged under stromal microenvironment. Interestingly, terminal (executioner) CASP3 (median=1.45; p=0.0319) and CASP 6 (median=1.33; p=0.0196) transcripts were significantly increased (Table 4).

Among four groups that are most affected, TNFR super family was one (Figure 18). TNFRSF family members analyzed were TNF, TNFRSF10A (DR4; Trail Receptor 1), TNFRSF10B (DR5; Trail Receptor 2), TNFRSF1A (Cd120a; TNFR1), TNFRSF1B (Cd120b; TNFR2), TNFRSF21 (DR6), TNFRSF25 (DR3; APO3), TNFSF10 (Trail), LTA (Lymphotoxin Alpha), LTB (Lymphotoxin beta), FAS, FASLG (Fas ligand). Interestingly, transcripts of TNFRSF1B (TNF receptor 2), which function as activator of anti-apoptotic signaling, were decreased at short (p=0.0014) and long (p=0.0054) incubations. Also transcripts of death receptors TNFRSF10A (DR4; p=0.0018), TNFRSF21 (DR6; p=0.0161) increased, and transcripts of pro-apoptotic ligands such as TNF and FAS increased on day 1 but unchanged at longer incubations (Table 4).

Table 4: Top hits of apoptotic transcripts in CLL cells impacted by stroma co-culturing

Function / family / Domain	Day 1	Median	P Value
CARD proteins family	NALP1	1.55	0.0012
TNFR Super family	TNFRSF1B	0.72	0.0014
TNFR Super family	TNFRSF10A	1.59	0.0018
Bcl-2 family	BCL2A1	1.66	0.0050
Bcl-2 family	BCL2L11	1.89	0.0054
ENTH domain / Pseudo DED	ESRRBL1	1.27	0.0059
Death Domain family	FADD	1.41	0.0107
Death Domain family	CFLAR	1.21	0.0125
ENTH domain / Pseudo DED	HIP1	1.67	0.0136
Death Domain family	DEDD2	1.28	0.0144
Bcl-2 family	BCL2	1.23	0.0150
TNFR Super family	TNFRSF21	2.63	0.0161
Death Domain family	TRADD	1.31	0.0171
NFkB proteins family	NFKBIA	1.38	0.0178
Bcl-2 family	BCL2L1	2.01	0.0179
Caspase proteins family	CASP6	1.33	0.0196
TNFR Super family	FAS	1.35	0.0197
Death Domain family	DEDD	1.19	0.0262
Caspase proteins family	CASP3	1.45	0.0319
TNFR Super family	TNF	1.34	0.0389
Bcl-2 family	BAD	1.33	0.0394
Bcl-2 family interaction	BBC3	1.32	0.0487

Bcl-2 family	
TNFRSF Family	
DED family	
NF-kB family	
Other families	

Function / family / Domain	Day 3	Median	P value
NFkB proteins family	NFKBIE	0.82	0.0003
Bcl-2 family	BCL2L1	0.72	0.0020
TNFR Super family	TNFRSF1B	0.71	0.0054
Inhibitors of IAPs	DIABLO	0.72	0.0301
NFkB proteins family	NFKBIZ	0.82	0.0310
NFkB proteins family	NFKB1	0.73	0.0338
Bcl-2 family	PMAIP1	0.66	0.0360
NFkB proteins family	NFKB2	0.78	0.0568

Figure 13: Heat map of stroma-induced changes in transcript levels of Bcl-2 family members

CLL cells were cultured in either suspension or stroma for 12 hours, 1 day or 3 days and were collected for real-time PCR array experiments. Changes in transcript levels of Bcl-2 family members are presented as heat map. Column is a gene name and row is a sample, and represents changes in expression level of transcript in CLL under stromal microenvironment. Fold change of transcripts were normalized with expressions in control cultures. Hierarchical clustering created using Agilent Genespring 11.0.1.

Figure 14: Heat map of stroma-induced changes in transcript levels of NF-kB family members

CLL cells were cultured in either suspension or stroma for 12 hours, 1 day or 3 days and were collected for real-time PCR array experiments. Changes in transcript levels of NF-kB family members are presented as heat map. Column is a gene name and row is a sample, and represents changes in expression level of transcript in CLL under stromal microenvironment. Fold change of transcripts were normalized with expressions in control cultures. Hierarchical clustering created using Agilent Genespring 11.0.1.

Figure 15: Heat map of stroma-induced changes in transcript levels of IAP family members

CLL cells were cultured in either suspension or stroma for 12 hours, 1 day or 3 days and were collected for real-time PCR array experiments. Changes in transcript levels of IAP family members are presented as heat map. Column is a gene name and row is a sample, and represents changes in expression level of transcript in CLL under stromal microenvironment. Fold change of transcripts were normalized with expressions in control cultures. Hierarchical clustering created using Agilent Genespring 11.0.1.
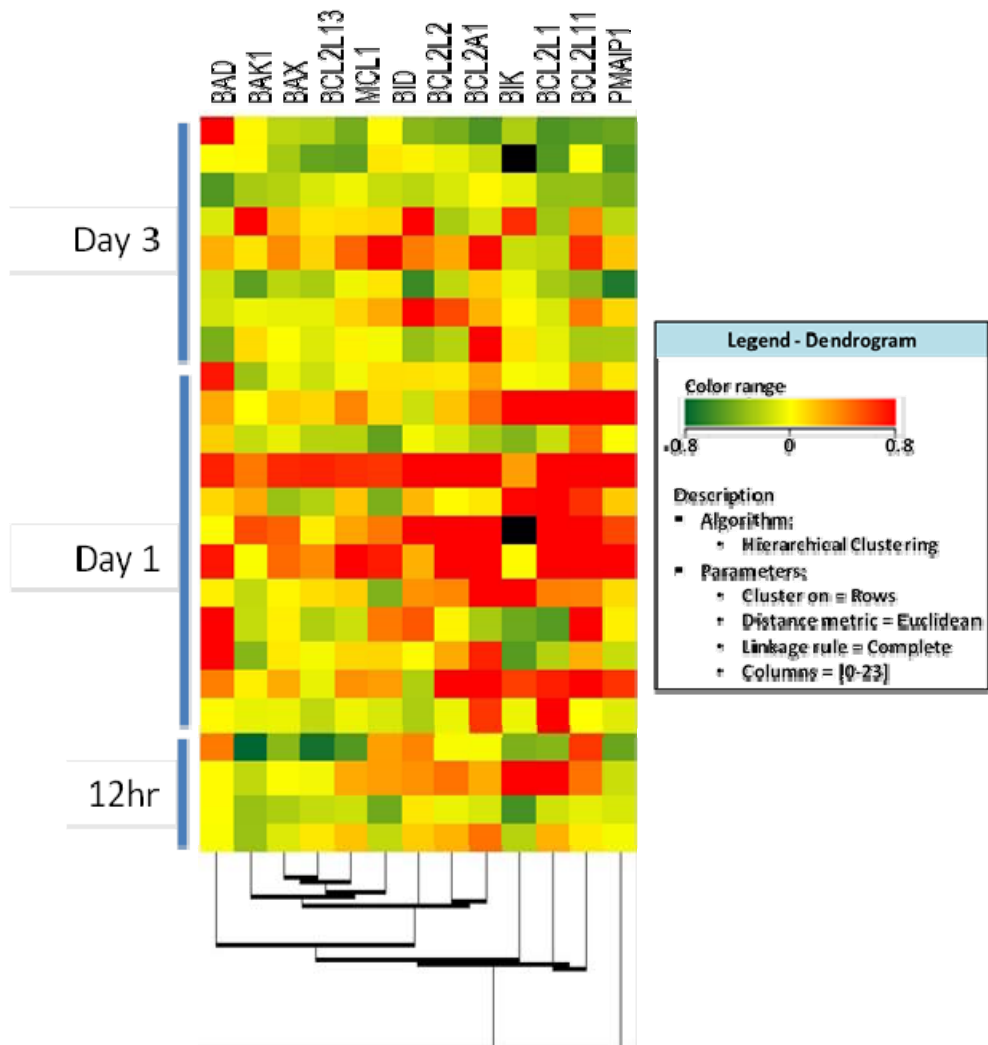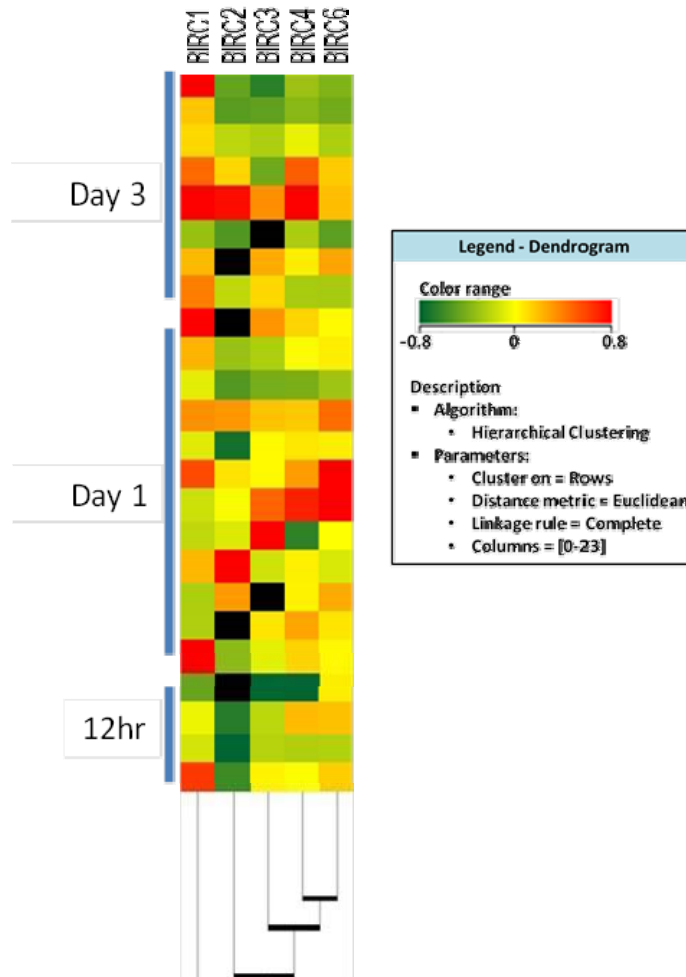
Figure 16: Heat map of stroma-induced changes in transcript levels of CARD and DED domain family members

CLL cells were cultured in either suspension or stroma for 12 hours, 1 day or 3 days and were collected for real-time PCR array experiments. Changes in transcript levels of CARD and DED family members are presented as heat map. Column is a gene name and row is a sample, and represents changes in expression level of transcript in CLL under stromal microenvironment. Fold change of transcripts were normalized with expressions in control cultures. Hierarchical clustering created using Agilent Genespring 11.0.1.
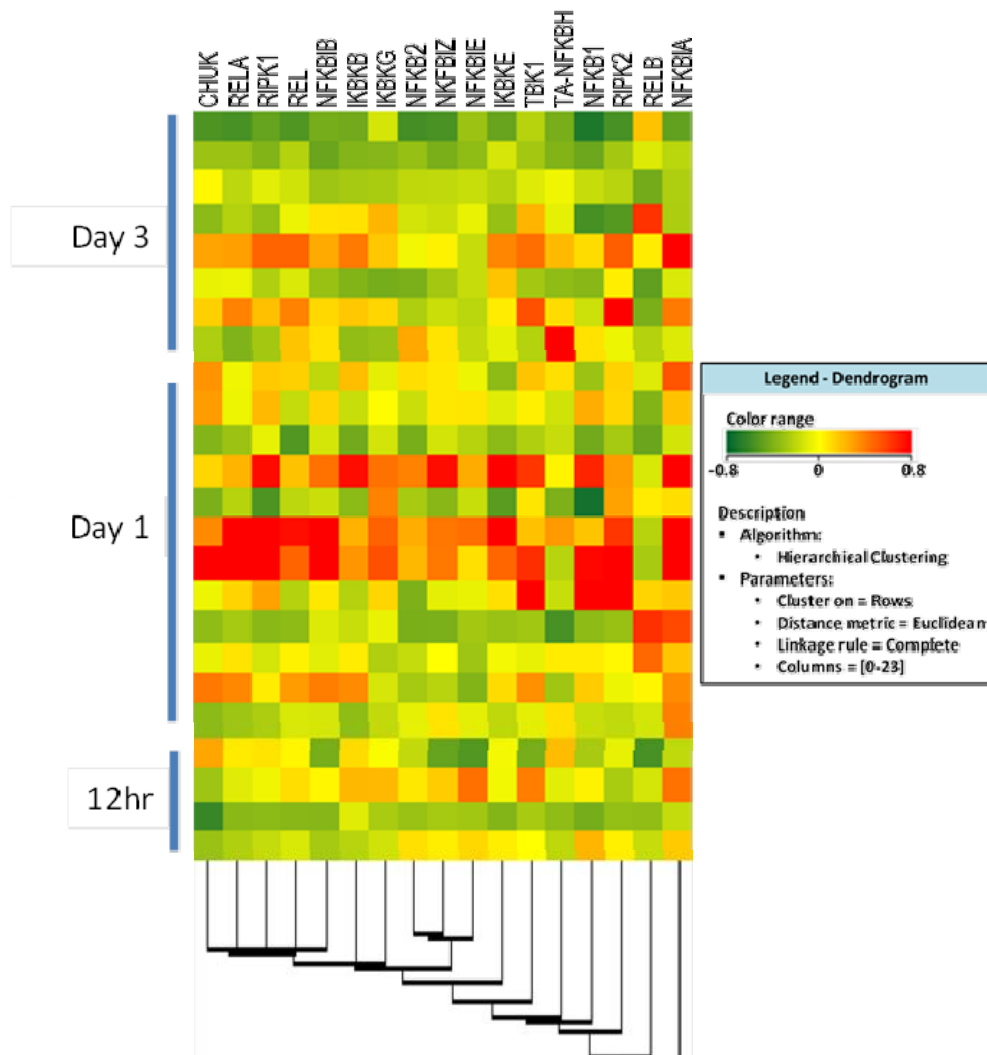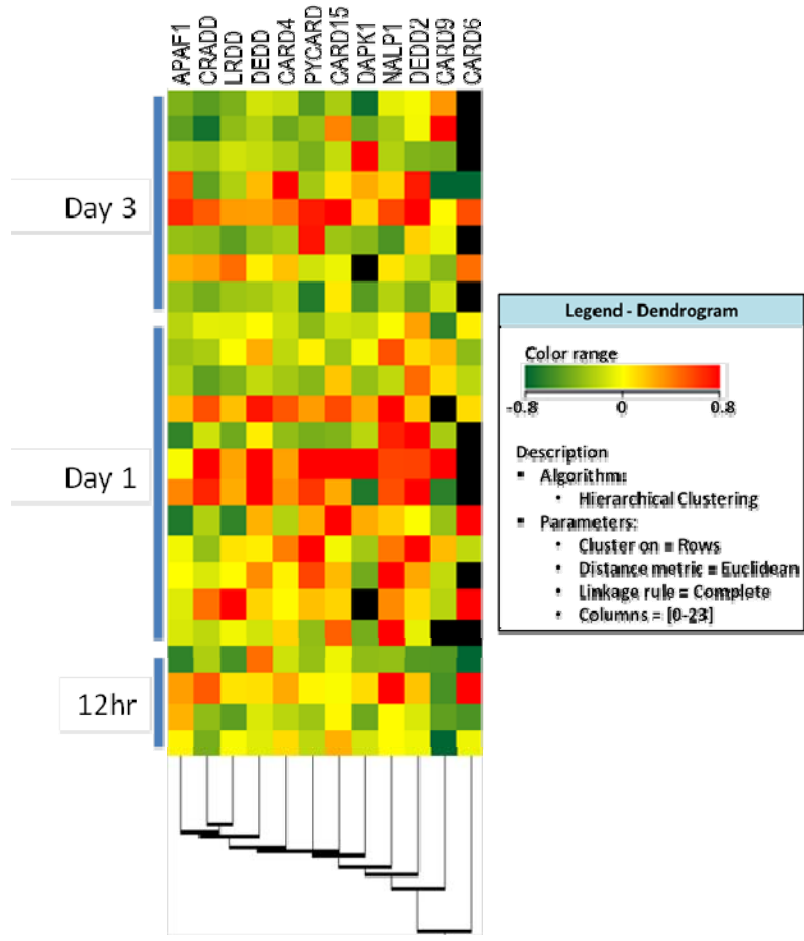
Figure 17: Heat map of stroma-induced changes in transcript levels of caspase family members

CLL cells were cultured in either suspension or stroma for 12 hours, 1 day or 3 days and were collected for real-time PCR array experiments. Changes in transcript levels of caspase family members are presented as heat map. Column is a gene name and row is a sample, and represents changes in expression level of transcript in CLL under stromal microenvironment. Fold change of transcripts were normalized with expressions in control cultures. Hierarchical clustering created using Agilent Genespring 11.0.1.

Figure 18: Heat map of stroma-induced changes in transcript levels of TNFRSF family members

CLL cells were cultured in either suspension or stroma for 12 hours, 1 day or 3 days and were collected for real-time PCR array experiments. Changes in transcript levels of TNFRSF family members are presented as heat map. Column is a gene name and row is a sample, and represents changes in expression level of transcript in CLL under stromal microenvironment. Fold change of transcripts were normalized with expressions in control cultures. Hierarchical clustering created using Agilent Genespring 11.0.1.
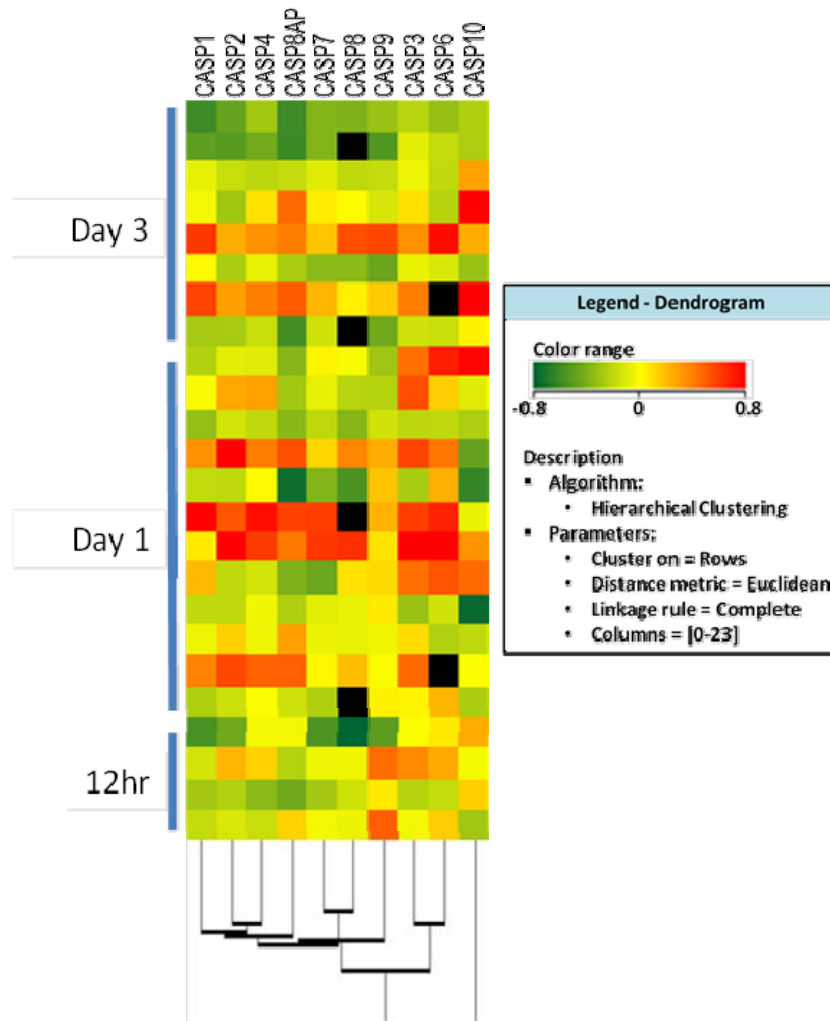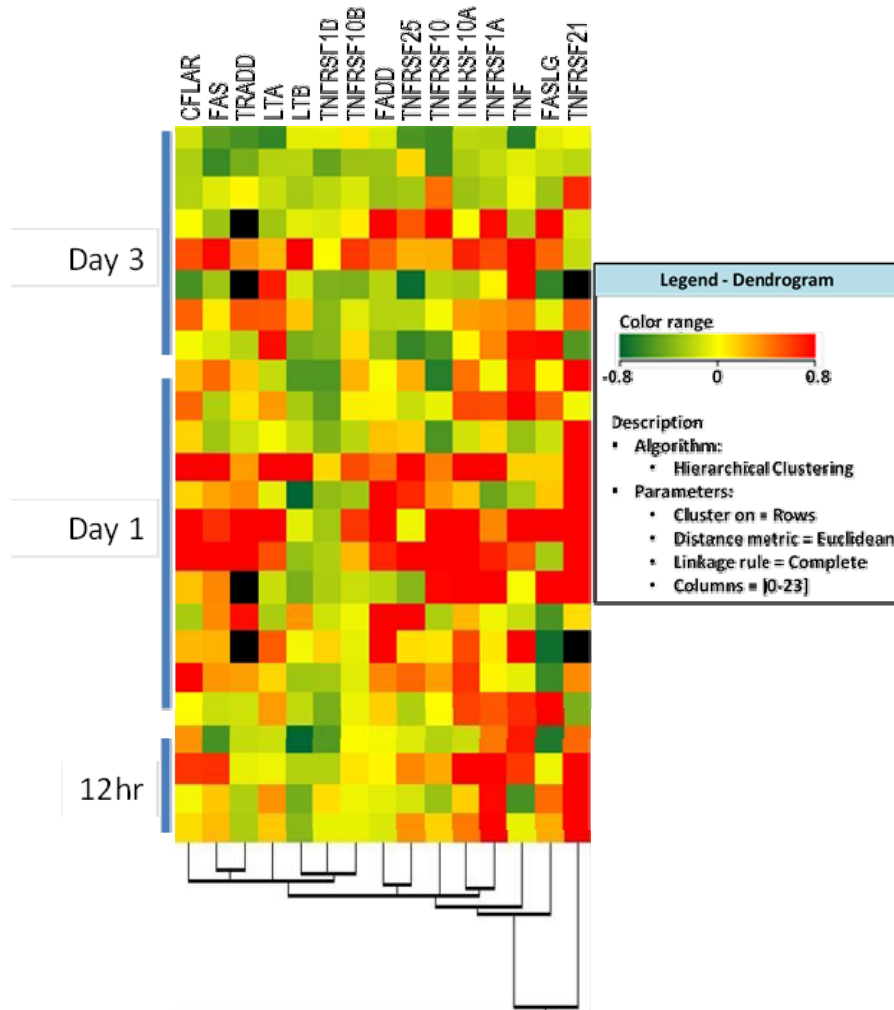
Aim 1.3: To analyze stromal-induced changes in Bcl-2 family mRNA and proteins in CLL cells

3.4: Stroma-induced changes in transcripts of Bcl-2 family members in CLL primary cells

Among seven groups of proteins, we studied Bcl-2 family of proteins in detail because of their significant role in the mechanism of CLL cell survival. Analysis of transcripts of apoptotic targets in CLL cells under stromal conditions demonstrated involvement of Bcl-2 family members both on 1 day (6/22 hits) and 3 days (2/8 hits) of cultures. Involvement of selected Bcl-2 family members in CLL survival under specific microenvironment has been reported before [37], [116]. Here, comprehensive analysis of changes in mRNAs (Figure 20, 21 and 22) and proteins (Figure 23, 24, and 25) of all Bcl-2 members (18) for 12 hours, day 1 and day 3 were carried out using real time RT-PCR array. Table 5 illustrates list of Bcl-2 family mRNAs analyzed and their expression in CLL cells. Real time PCR array microfluidic card contained 18 Bcl-2 family members, and of them, three were of BH3 only pro-apoptotic, nine were of multi-domain BH3 pro-apoptotic and six were of anti-apoptotic proteins members. Figure 19 illustrates schematic representation of pro- and anti-apoptotic Bcl-2 family members. Out of 18 Bcl-2 family members analyzed, 13 were expressed endogenously in CLL cells, while 5 of them (BCL2L10 (BCL-B) (anti-apoptotic member) and BCL2L14, BCL2L13 (BCL-RAMBO), HRK, and BOK (pro-apoptotic member) were below level of detection in most samples (Table 5).

Stroma induced modest increase (1-1.25 fold) in transcripts of anti-apoptotic members' MCL-1, BCL1A1, BCL2L1 (BCL-XL), BCL-2 and BCL2L2 (BCL-W) in CLL cells within 12 hours of co-culturing (Figure 20 A-E). On day 1, stroma-induced 1.3 to 2.1 fold increase (Figure 20) with maximum increase in BCL-XL mRNA levels and minimum or no change in BCL-W mRNA levels.

Interestingly, by day 3 incubations, stroma-induced transcripts were returned to basal level as those of in suspension CLL cells (Figure 20 A-E).

Stromal cells induced decrease in transcripts of Bcl-2 family pro-apoptotic members, BAK (median fold change 0.62; p=0.02) and BAX (median fold change 0.82; p>011; insignificant) at 12 hours, and remained unchanged on day 1 and day 3 co-culturing (Figure 21A-B). As described above, Bok was below level of detection. Anti- and pro-apoptotic have opposing functions, and therefore differential response to co-cultures would be predicted. However, stroma increased BH3 only members on day 1, but returned to basal level similar to that of anti-apoptotic members. BH3 only pro-apoptotic members, PUMA (p=0.0487), NOXA (p=0.0505), BAD (p=0.0394), BIM (0.0054) expression were increased on day 1 and decreased on day 3 (Figure 22A, B, D, F respectively). Moreover, mRNA expressions of BH3 only pro-apoptotic Bcl-2 family members, BID (Figure 22C) and BIK (Figure 22E) were unchanged under stromal microenvironment.

3.5: Stroma-induced changes in protein levels of Bcl-2 family members

With the outcome of stroma-induced variability in transcripts of Bcl-2 family members, we have analyzed protein levels of Bcl-2 family members (Figure 23, 24, and 25). Immunoblot analysis of anti-apoptotic Bcl-2 family proteins of one representative patient data is presented as Figure 23A. Stromal microenvironment induced either increased or stable expressions of anti-apoptotic proteins Bcl-2, Bcl-x_L, Bfl-1, Mcl-1, and Bcl-w in CLL cells on day 1 and day 3 (Figure 23B-F; n=5). Expressions of Bcl-2, Bcl-x_L, Mcl-1, and Bcl-w were increased in CLL cells on day 3. All patients' samples analyzed for viability showed sustainability in presence of stromal microenvironment except patient # 31 (data not shown). Consistently, expressions of almost all anti-apoptotic Bcl-2 family proteins were decreased on day 1 in this sample (data not shown).

Pro-apoptotic Bcl-2 family proteins were also analyzed. Representative immunoblot for Bax, multidomain pro-apoptotic Bcl-2 family protein, is presented (Figure 24A). Similar to transcript data, Bax protein did not change on day 1 and day 3 stromal co-culturing (Figure 24B). Bak protein levels were not analyzed in this study. For BH3-only pro-apoptotic Bcl-2 family proteins, representative immunoblots are showed in Figure 25A. Bid protein did not change (Figure 25B; n=4) and Bik decreased on day 1 (Figure 25C ; n=4; P=0.043;). 3 out of 4 samples showed increase in Bad protein levels on day 1 but were unchanged on day 3 (Figure 25D). Moreover, expression of Puma decreased slightly on day 1 (n=4, p=0.072) and remained unchanged until day 3 (Figure 25H). In addition, Noxa protein (Figure 25G), and short (Figure 25E) and long (Figure 25F) isoforms of Bim proteins were stable on both day 1 and day 3 co-cultures.

Figure 19: Schematic representation of pro- and anti- apoptotic Bcl-2 family members

BH3 domains for anti-apoptotic (6), pro-apoptotic multidomain (3) and pro-apoptotic BH3 only (9),

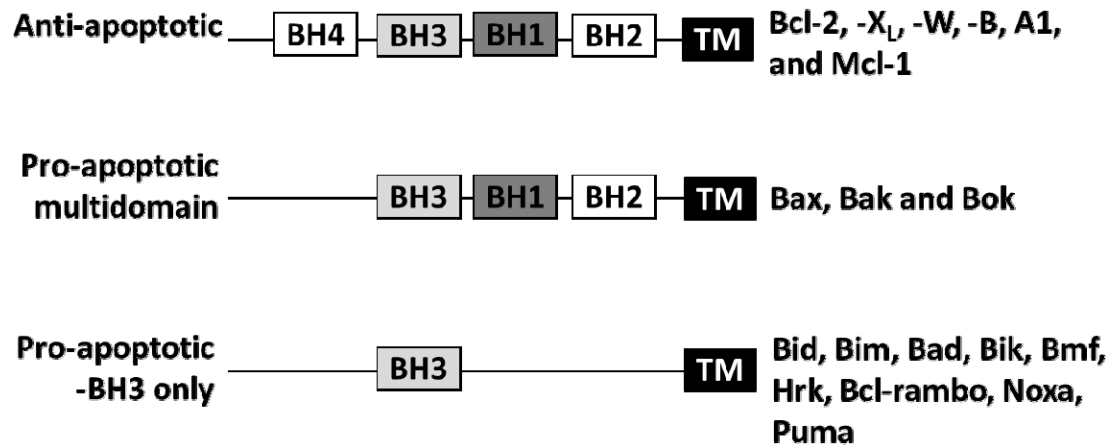Bcl2 family proteins are represented through schematics.

Table 5: Expression of Bcl-2 family mRNAs in CLL lymphocytes

Table I. Bcl-2 family genes and proteins on human apoptotic array microfluidic card and their detection in CLL lymphocytes[*].

	Gene names	Protein names	Function	Detected in array[†]	Detected total n[‡]
1	BCL-2	Bcl-2	Anti-apoptotic	Y	12/12
2	BCL2A1 (BFL-1)	Bfl-1	Anti-apoptotic	Y	12/12
3	BCL2L1 (BCL-XL)	Bcl-x$_L$	Anti-apoptotic	Y	12/12
4	MCL-1	Mcl-1	Anti-apoptotic	Y	12/12
5	BCL2L10 (BCL-B)	Bcl-b	Anti-apoptotic	N	3/12
6	BCL2L2 (BCL-W)	Bcl-w	Anti-apoptotic	Y	12/12
7	BAK1 (BAK)	Bak	Pro-apoptotic multi-domain	Y	12/12
8	BAX	Bax	Pro-apoptotic multi-domain	Y	12/12
9	BOK	Bok	Pro-apoptotic multi-domain	N	2/12
10	BCL2L11 (BIM)	Bim	Pro-apoptotic BH3-only	Y	12/12
11	BCL2L13	Bcl-rambo	Pro-apoptotic BH3-only	N[§]	12/12
12	BCL2L14	Bcl-g	Pro-apoptotic BH3-only	N	2/12
13	BAD	Bad	Pro-apoptotic BH3-only	Y	12/12
14	BBC3 (PUMA)	Puma	Pro-apoptotic BH3-only	Y	12/12
15	HRK	Hrk	Pro-apoptotic BH3-only	N	6/12
16	PMAIP1 (NOXA)	Noxa	Pro-apoptotic BH3-only	Y	12/12
17	BID	Bid	Pro-apoptotic BH3-only	Y	12/12
18	BIK	Bik	Pro-apoptotic BH3-only	Y	12/12

CLL, chronic lymphocytic leukemia; PCR, polymerase chain reaction.
[*]Bcl-2 family members analyzed through real-time PCR using microarray fluidic card (Applied Biosystems).
[†]Y, detected in all samples analyzed ($n = 12$); N, not detected in one or more samples analyzed.
[‡]See text for respective mRNAs for more details.
[§]Bcl2L13 detection was above cycle 35, hence it was not considered for calculation.

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Figure 20: Comparison of transcript levels of anti-apoptotic Bcl-2 family members between CLL cells in suspension and stromal co-cultures

CLL cells were cultured in suspension or co-cultured on stroma for 3 days (NKTert). Using real time PCR array (micro fluidic card), individual transcript level of anti- apoptotic Bcl-2 family members were determined for 12 hours, day 1 and day 3 (A-E). Expressions of each transcript level in CLL cells on stroma were normalized to respective suspension cultures. Each dot in graph represents fold change in mRNA expression of respective Bcl-2 family member for each CLL patient sample. Statistical analysis was performed using one sample t test (hypothetical value 1; *P<0.05).

Figure 21: Comparison of transcript levels of multidomain pro-apoptotic Bcl-2 family members between CLL in suspension and stromal co-cultures

CLL cells were cultured in suspension or co-cultured on stroma for 3 days (NKTert). Using real time PCR array (micro fluidic card), individual transcript level of multidomain pro-apoptotic Bcl-2 family members were determined for 12 hours, day 1 and day 3 (A-B). Expressions of each transcript level in CLL cells on stroma were normalized to respective suspension cultures. Each dot in graph represents fold change in mRNA expression of respective Bcl-2 family member for each CLL patient sample.   Statistical analysis was performed using one sample t test (hypothetical value 1; *P<0.05).

Figure 22: Comparison of transcript levels of BH3 only pro-apoptotic Bcl-2 family members between CLL in suspension and stromal co-cultures

CLL cells were cultured in suspension or co-cultured on stroma for 3 days (NKTert). Using real time PCR array (micro fluidic card), individual transcript level of BH3 only pro-apoptotic Bcl-2 family members were determined for 12 hours, day 1 and day 3 (A-F). Expressions of each transcript level in CLL cells on stroma were normalized to respective suspension cultures. Each dot in graph represents fold change in mRNA expression of respective Bcl-2 family member for each CLL patient sample. Statistical analysis was performed using one sample t test (hypothetical value 1; *P<0.05).
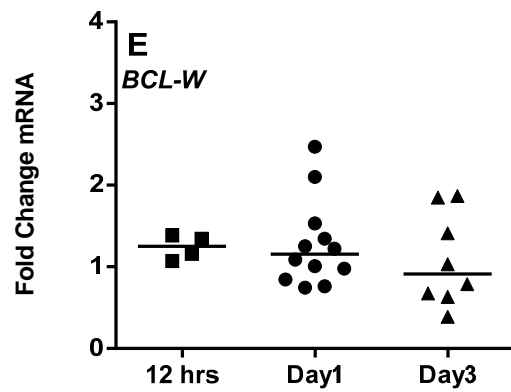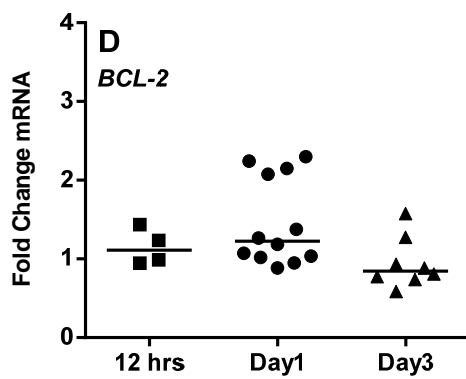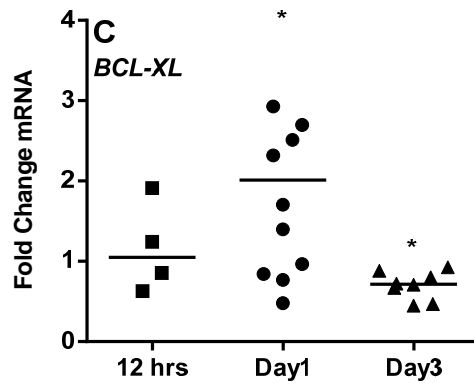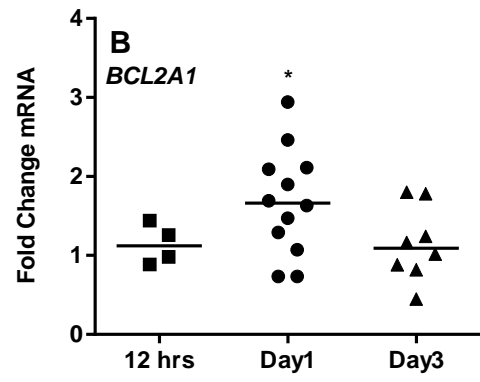
^Outlier was ignored

Figure 23: Comparison of protein levels of anti-apoptotic Bcl-2 family members between CLL in suspension and stromal co-cultures

CLL cells were cultured in suspension or co-cultured on stroma for 3 days (NKTert cells). Using immunoblot assays, individual protein levels of anti-apoptotic Bcl-2 family members were determined for day 1 and day 3. Representative immunoblots for anti-apoptotic proteins (A) and quantitation of immunoblots of all patient samples analyzed (B-F) are provided. Expressions of each member proteins level in CLL cells on stromal co-cultures were normalized to expressions in respective suspension cultures. Each dot in graph represents stromal cell coincubation-induced fold change in protein expression of respective Bcl-2 family member per one CLL patient sample. Statistical analysis was performed using one sample t test (hypothetical value 1; *P<0.05).
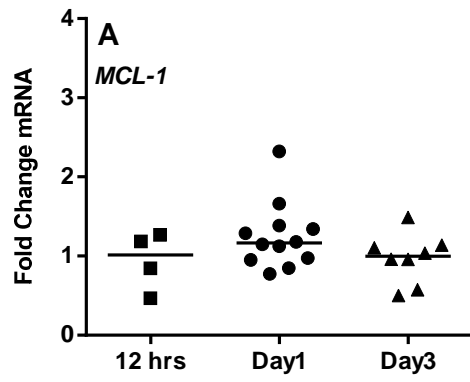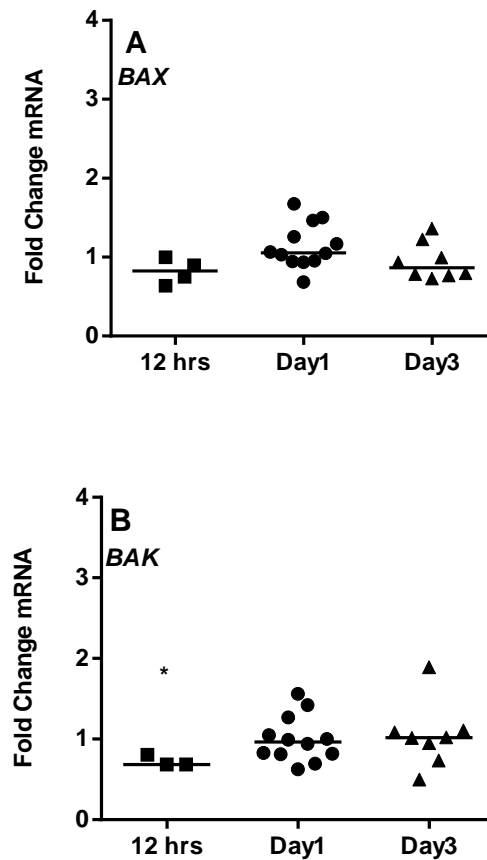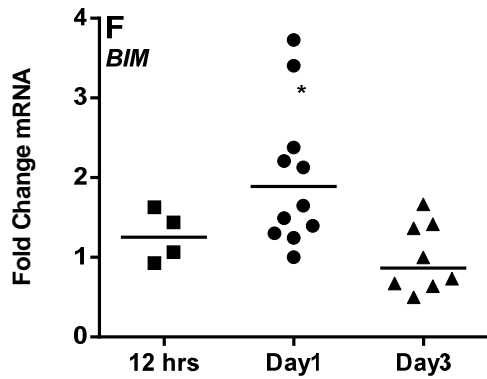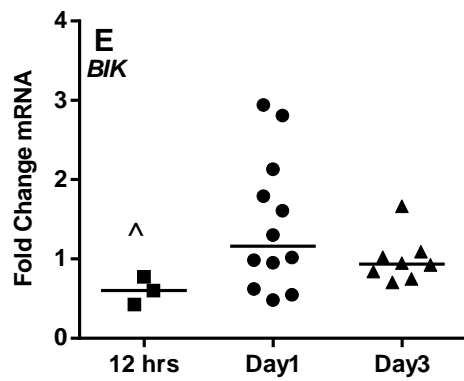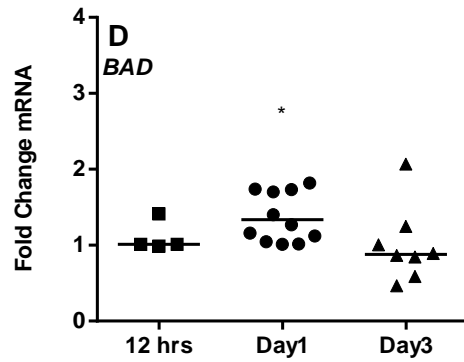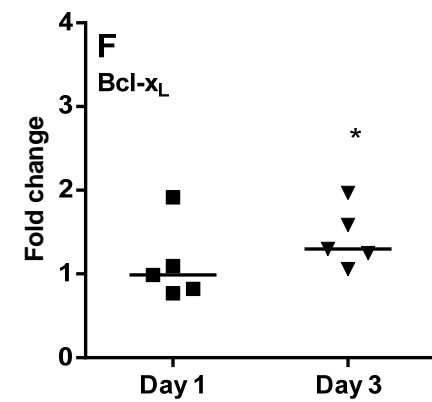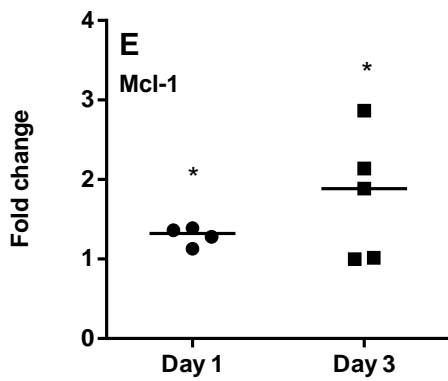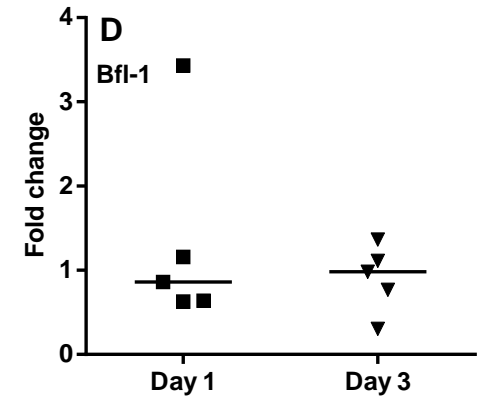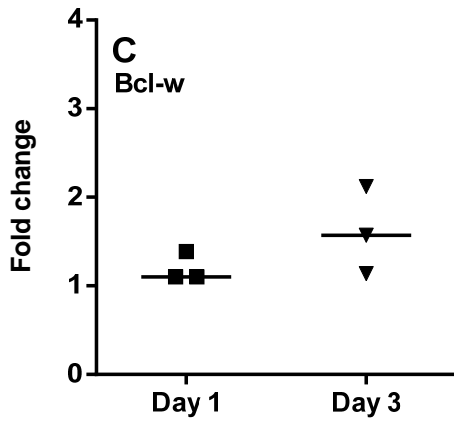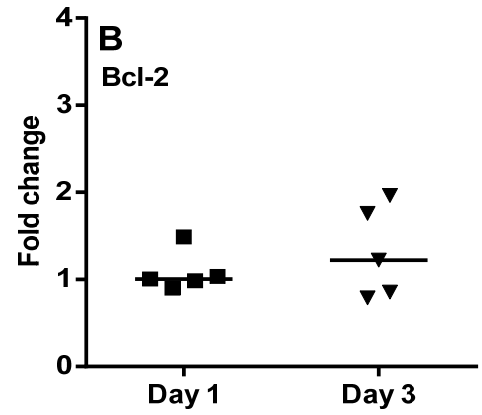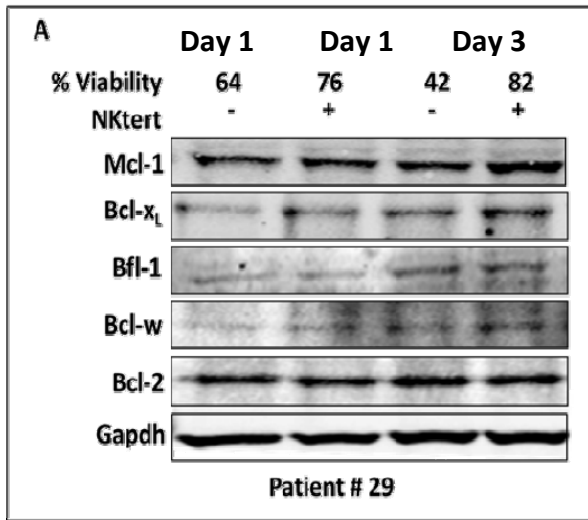
Figure 24: Comparison of protein levels of multidomain pro-apoptotic Bcl-2 family members between CLL in suspension and stromal co-cultures

CLL cells were cultured in suspension or co-cultured on stroma. Using immunoblot assays, protein level of multidomain pro-apoptotic Bcl-2 family member Bak was determined. Representative immunoblots for multidomain pro-apoptotic Bcl-2 family Bax proteins (A) and quantitation of immunoblots of all patients analyzed (B) are provided. Expressions of Bax protein levels in CLL cells on stromal co-cultures were normalized to expressions in respective suspension cultures. Each dot in graph represents stromal cell coincubation-induced fold change in protein expression of respective Bcl-2 family member per one CLL patient sample.
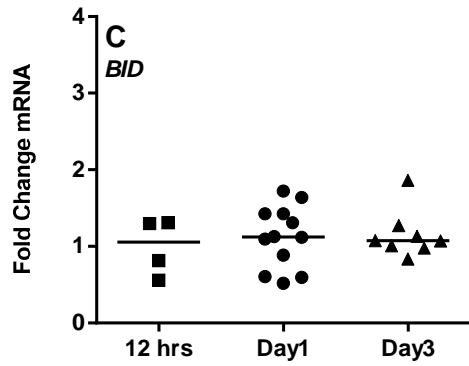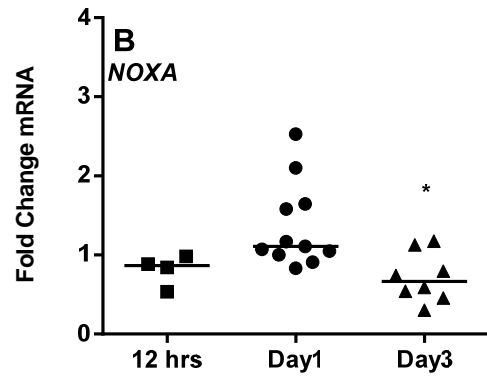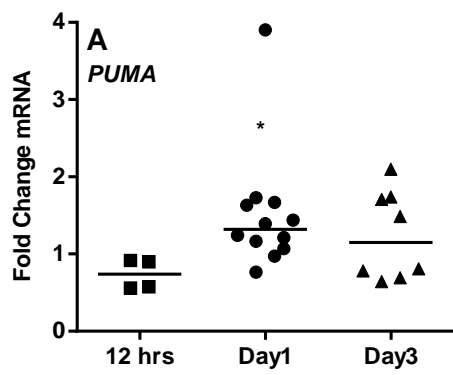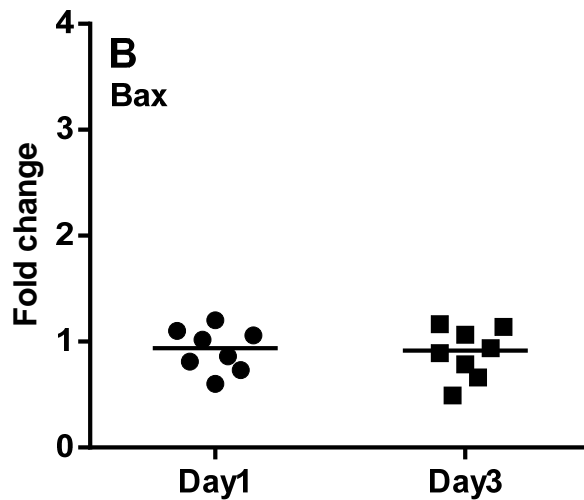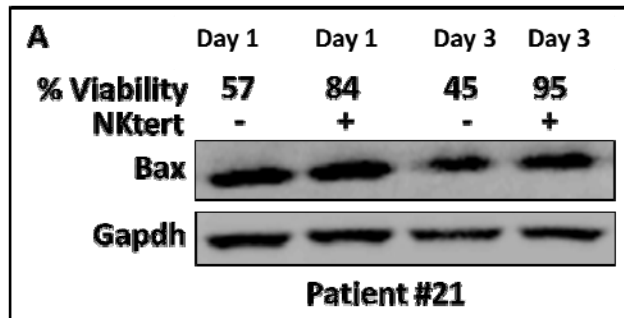
Figure 25: Comparison of protein levels of BH3 only pro-apoptotic Bcl-2 family members between CLL in suspension and stromal co-cultures

CLL cells were cultured in suspension or co-cultured on stroma. Using immunoblot assays, individual protein level of BH3 only pro-apoptotic Bcl-2 family members were determined. Representative immunoblots for pro-apoptotic BH3 only Bcl-2 family proteins (A) and quantitation of immunoblots of all patients analyzed (B-H) are provided. Expressions of each member proteins level in CLL cells on stromal co-cultures were normalized to expressions in respective suspension cultures. Each dot in graph represents stromal cell coincubation-induced fold change in protein expression of respective Bcl-2 family member per one CLL patient sample. Statistical analysis was performed using one sample t test (hypothetical value 1; *P<0.05).

A

	Day 1	Day 1	Day 3	Day 3
% Viability	57	84	45	95
NKtert	-	+	-	+

Bid

Bim$_{EL}$
Bim$_{L/S}$

Bad

Noxa

Gapdh

Patient # 21

% Viability	81	86	86	98
NKtert	-	+	-	+

Puma

Gapdh

Patient # 16

% Viability	75	88	-	-
NKtert	-	+	-	+

Bik

Gapdh

Patient # 32

B
Bid
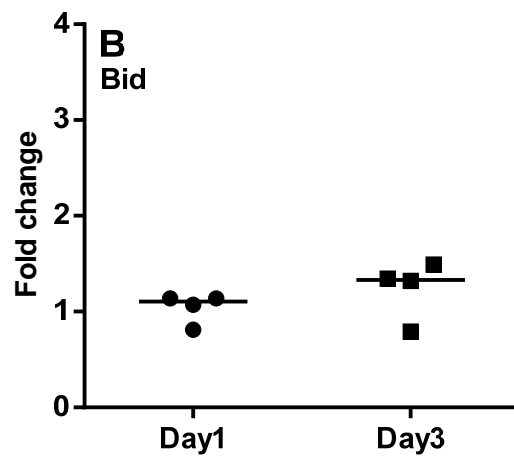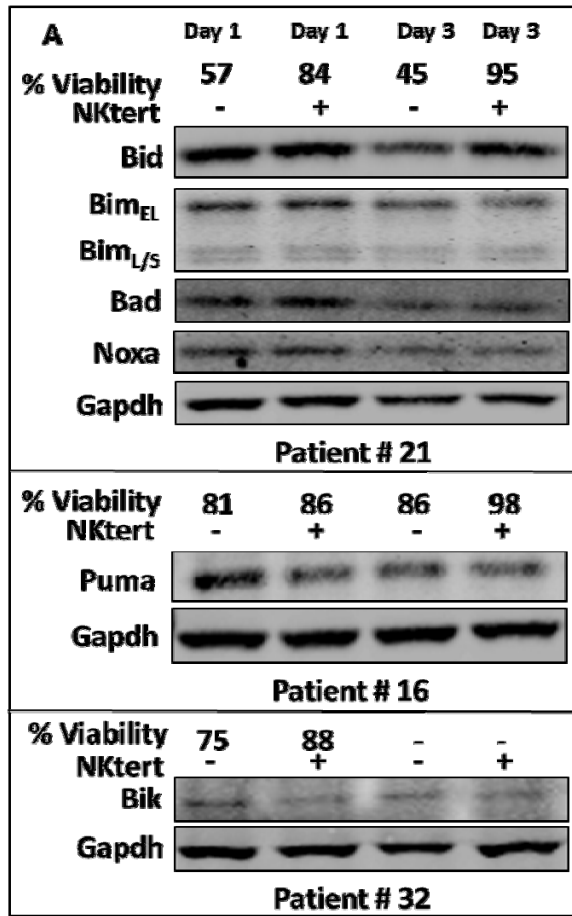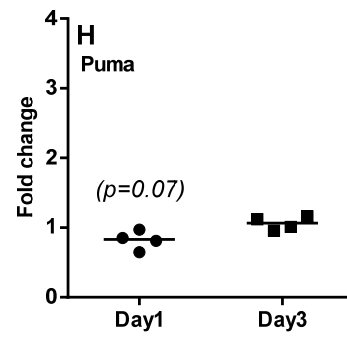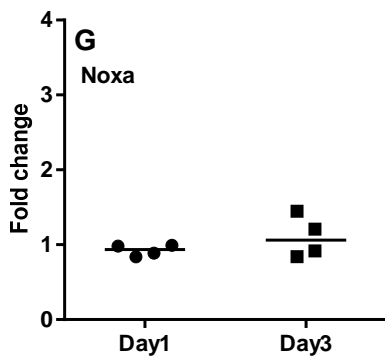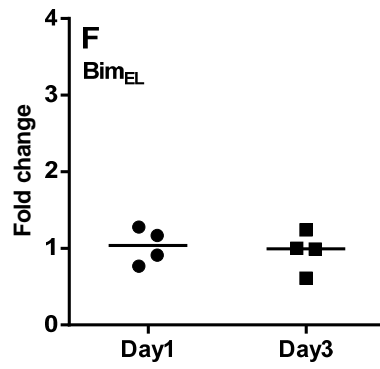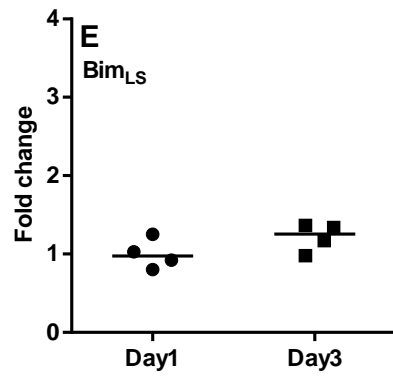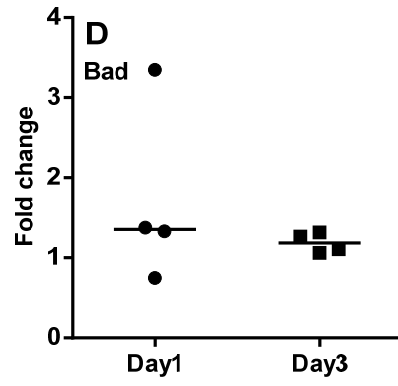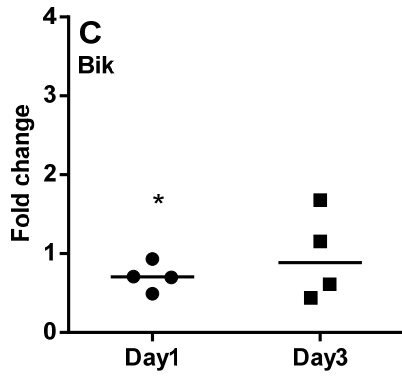
CHAPTER 4: RESULTS: Therapeutic activation of procaspases in CLL

Hypothesis II

Based on survival advantage of CLL cells due to endogenous overexpression of anti-apoptotic proteins, their induction by microenvironment, role of caspases in CLL cell death, and actions of L14R8 in converting inactive procaspase-3 to caspase-3, I hypothesized that L14R8 will directly activate the functional ability of executioner caspases and should overcome the checkpoints of cell death to trigger apoptosis in CLL lymphocytes.

This section of results (chapter 4) provides all data obtained for hypothesis II

Aim 1.1: To investigate biological consequences of L14R8 treatment in CLL primary samples

4.1: CLL lymphocytes express executioner caspases

Proposed mechanism of action of L14R8 is through activation of executioner procaspases. Hence, presence of executioner procaspases is a primary requisite for drug's action. Real-time PCR microarray analysis, showed expression of all initiator procaspases (-2, -8, -9, and -10) and all executioner procaspases (-3, -6, and -7) (Table 6). Among inflammatory caspases, only procaspase-1 and procaspase-4 were detected in all CLL samples tested (n=12), while procaspase-5 was detected in 4/12 samples (Table 6).

At protein level, expressions of executioner procaspases were compared between healthy PBMCs and malignant CLL lymphocytes using immunoblots. Also expressions of executioner procaspases-3 and -7 were compared between purified normal B cells and malignant CLL lymphocytes (Figure 26). Procaspase-3 (Figure 27A) and -7 (Figure 27B) bands were confirmed. For

all 3 procaspases-3, -6, and -7, PBMCs had spontaneous apoptosis as active caspases bands were detected, while patient CLL lymphocytes (mostly B-cells) showed either absence or low levels of active proteins, indicating resistance to apoptosis (Figure 26).

4.2: L14R8 treatment induced apoptosis in CLL lymphocytes

CLL lymphocytes were incubated with DMSO alone or L14R8 for 24 hours at various concentrations (Figure 28A-F). L14R8 induced dose-dependent apoptosis in CLL lymphocytes (IC_{50}~10 μM, n=26) (Figure 28A). Moreover, individual graphs of different concentration of L14R8 represented each patient samples response (Figure 28B-F). DMSO treated samples with <40% viability were not included (n=2). When CLL cells were incubated with 10μM L14R8 for 2, 4, 6, 8, 10 hours, apoptosis was detected as early as 6 hours, however was significant at 10 hours (n=5; p<0.05; Figure 29).

To evaluate therapeutic index of L14R8, pure normal B cells, freshly isolated PBMCs, and CLL patient lymphocytes were incubated with 10μM L14R8 for 24 hours. L14R8-induced apoptosis was 31% in normal B cells (range 19-53%), 18% (range 16-28%) in normal PBMCs and 64% (range 18-90%) in leukemic lymphocytes indicating selectivity of L14R8 towards CLL lymphocytes (Table 7).

4.3: L14R8-induced apoptosis in CLL cells in the presence of different microenvironment

As described earlier, sustained survival and chemoresistance in CLL cells associate with signals from microenvironment [37,147,148]. To evaluate, if L14R8 induced apoptosis is affected by microenvironment, CLL lymphocytes were co-cultured with NKTert (human bone marrow stromal) cells, in presence or absence of L14R8 for 24 hours. 10 μM L14R8 treatment induced apoptosis in CLL lymphocytes both in the absence (p=0.0237; n=4) and presence (p=0.0111; n=4) of stromal cells, albeit at lower rate in the presence of stromal cells (Figure 30). Other microenvironment

factors such as IL-6, IgM and CD40L were also evaluated. % viability in CLL lymphocytes co-incubated with IL-6, IgM and CD40L significantly reduced (for all 3 factors: p<0.0001; n=11) after L14R8 treatment compared to DMSO treatment. Importantly, % viability in L14R8 treated cells in the presence or absence of these factors was not significantly different (p=ns) indicating L14R8 induces apoptosis in CLL lymphocytes regardless of 3 microenvironment factors (Figure 31).

4.4: L14R8-induced apoptosis in CLL lymphocytes with respect to prognostic factors

To evaluate the impact of various prognostic factors on L14R8-induced apoptosis, % viability of CLL lymphocytes with 10 μM L14R8 treatment were replotted. CLL lymphocytes treated with L14R8 were more sensitive to Rai stage '0' (22% viability; p=0.0094) compared to Rai stage '1' (38% viability), '2' (41% viability), '3' (54% viability), and '4' (40% viability), hence these were not significantly different than IC_{50} value (for stage 2,3,4; p=ns; Figure 32A). Trisomy-12 samples were more resistant (median: 54% viability) to L14R8 treatment compared to deletions in chromosome 13 at q14 (38% viability) and deletions in chromosome 17p (39% viability). Samples analyzed for deletions in chromosome 11 at q22 or q23 were not sufficient to conclude effect of this alteration (Figure 32B). Separate analysis of 17p deletion negative or positive samples shown as Figure 32C suggested no apparent effect of this cytogenetic alteration (p=ns). CLL lymphocytes with mutated (36% viability) and unmutated (39% viability) IgVH status, responded similarly to L14R8 treatment (p=ns) (Figure 32D). Similarly, ZAP70 status (ZAP70 positive, 39% viability and ZAP70 negative, 39% viability; Figure 32E), prior fludarabine treatment exposure (presence (31% viability) or absence (40% viability); Figure 32F), and level of WBC in peripheral blood (Figure 32G), did not impact L14R8-mediated apoptosis (p=ns). Interestingly, higher expression of β_2 microglobulin (unit >4; 62% viability) showed resistance to L14R8 treatment compared to lower expression of β_2 microglobulin (unit <3; 24% viability) (p=0.0094) (Figure 32H).

Table 6: Expression of caspases mRNAs in CLL lymphocytes

Expressions of caspases transcripts in CLL lymphocytes were comprehensively analyzed using real time PCR array (microfluidic card) in 12 CLL patient samples. The presence of caspases transcripts were analyzed in CLL lymphocytes at 24 hours in suspension cultures.

Function[136]	Gene name	Protein name	+ve in samples / total samples
Apoptosis Initiator	CASP2	Caspase-2	12/12
Apoptosis Initiator	CASP8	Caspase-8	12/12
Apoptosis Initiator	CASP9	Caspase-9	12/12
Apoptosis Initiator	CASP10	Caspase-10	12/12
Apoptosis Effector	CASP3	Caspase-3	12/12
Apoptosis Effector	CASP7	Caspase-7	12/12
Apoptosis Effector	CASP6	Caspase-6	12/12
Pro-inflammatory, and pyroptosis	CASP1	Caspase-1	12/12
Pro-inflammatory, and pyroptosis	CASP4	Caspase-4	12/12
Pro-inflammatory, and pyroptosis	CASP5	Caspase-5	4/12
Keratinocyte differentiation	CASP14	Caspase-14	0/12

Figure 26: Comparisons of protein levels of procaspase-3, -6, and -7 between healthy donors' PBMCs/B cells and CLL lymphocytes

Comparisons of protein expressions of procaspase -3, -6, and -7 between peripheral blood monocyte cells (PBMCs) and CLL lymphocytes were carried out (A-C). Also expression of procaspase-3 and -7 proteins in purified normal B cells and CLL lymphocytes were carried out (D-E). Freshly obtained peripheral blood from healthy donors and CLL patients were used to isolate pure B cells, PBMCs and CLL lymphocytes respectively. Collected fresh cells were processed for immunoblot analysis and expression of procaspase-3 protein, Procaspase-6 protein and Procaspase-7 protein is presented. GAPDH is the loading control.
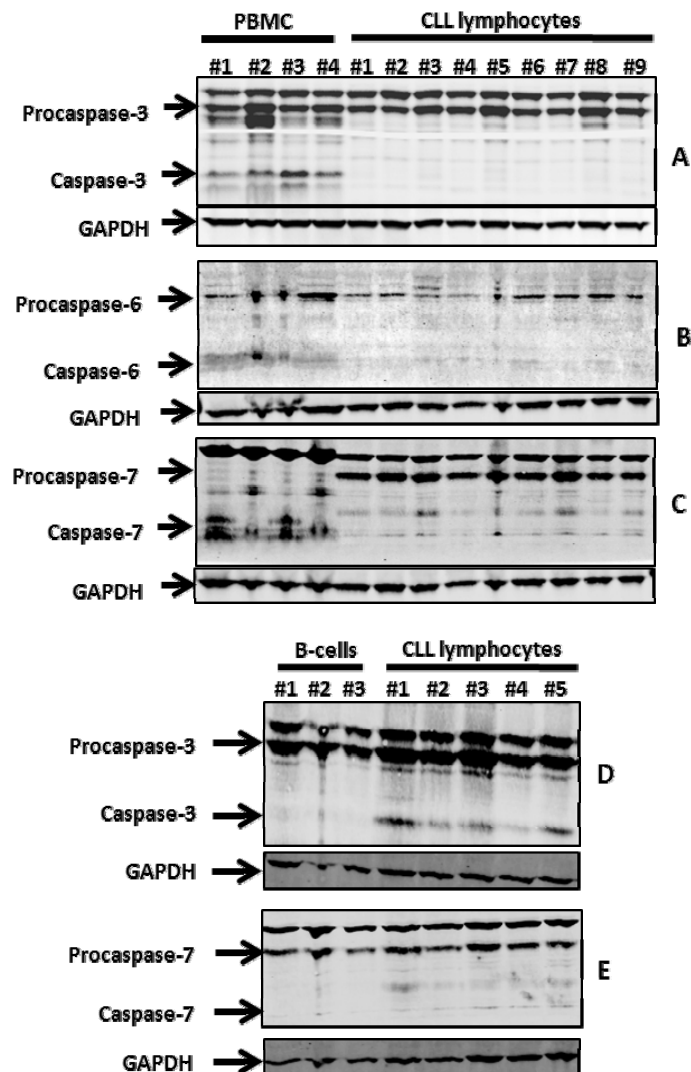
Figure 27: Confirmation of procaspase-3 and -7 bands

CLL lymphocytes treated with different concentration of L14R8 for 24 hours or with 10 µM L14R8

for 24 hours were processed for immunoblot analysis to confirm procaspase-3 (A) and procaspase-7

bands (B). Jurkat cells treated with staurosporine were used as positive (+ve) control and untreated

jurkat cells were used as negative control (–ve), which were commercially obtained from Cell

Signaling, Danvers, MA.
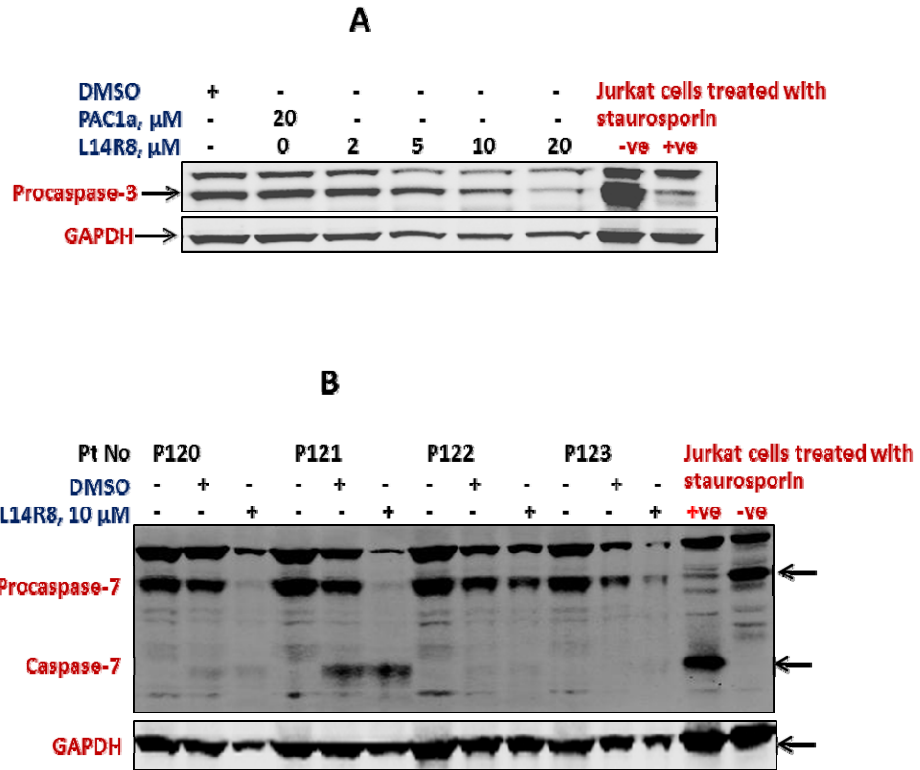
Figure 28: L14R8 induces cell death in CLL cells

CLL lymphocytes from peripheral blood of 26 patients were isolated and treated with 2, 5, 8, 10, 20, 30, 50 μM L14R8 for 24 hours. IC_{50} of L14R8 induced apoptosis was measured using exponential decay graph (A). The same data were plotted using linear graphs to visualize individual patient response (B-F). % cell death was measured by Annexin V/PI staining method.
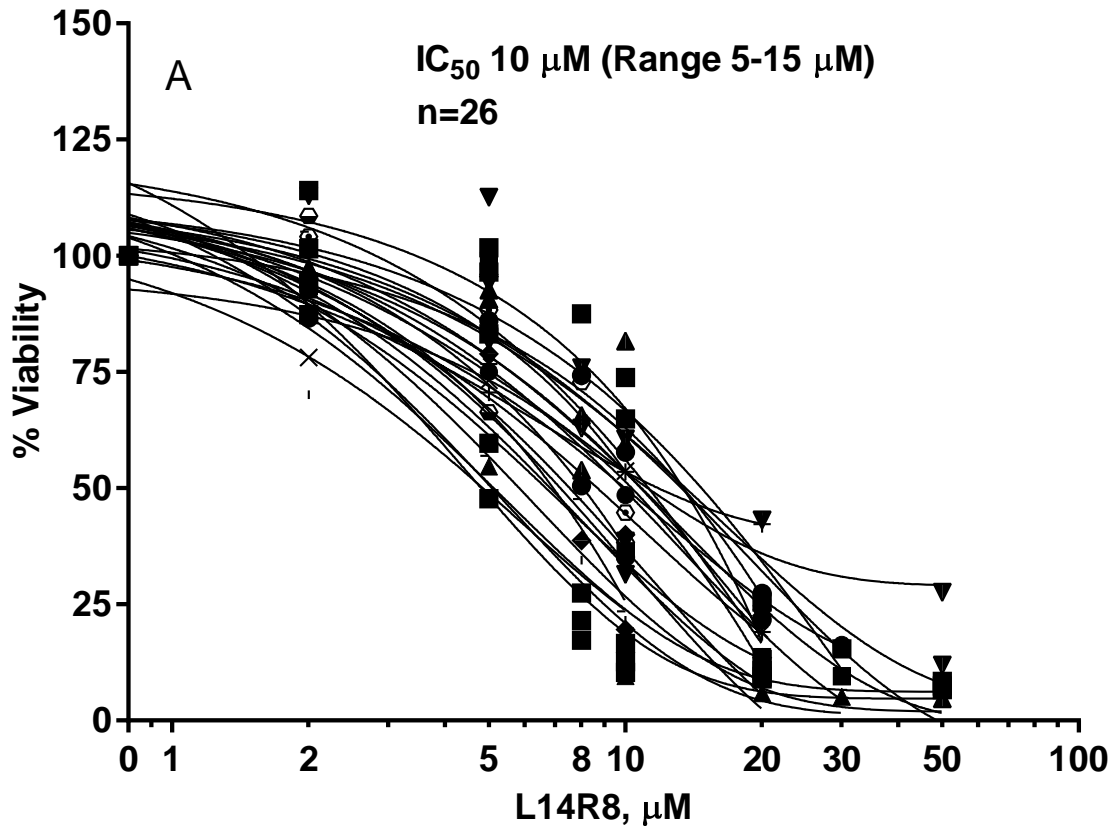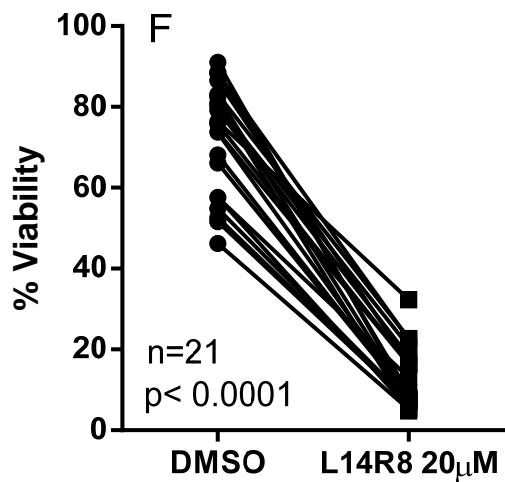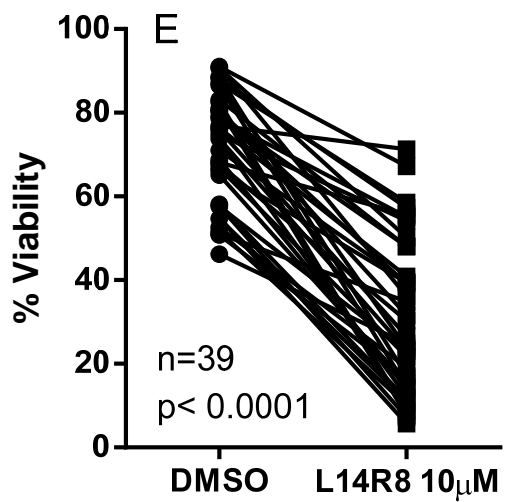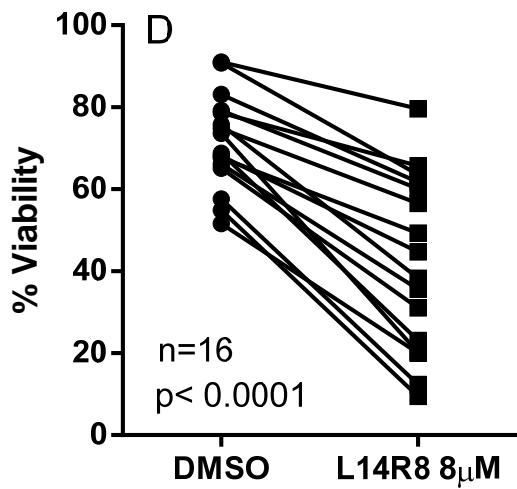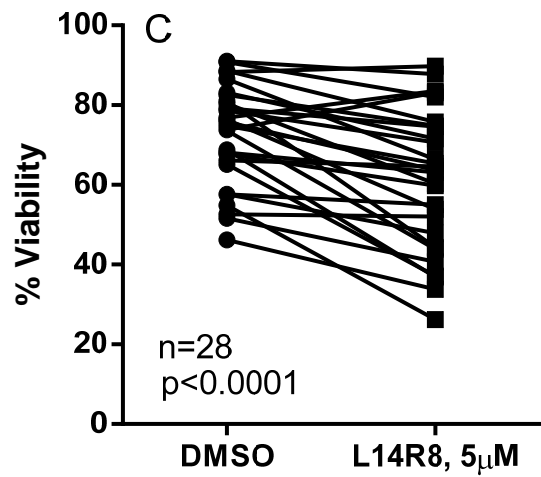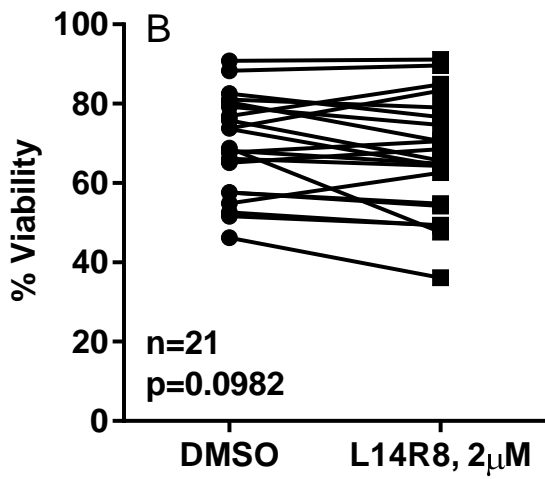
Figure 29: Effect of L14R8 shorter treatments in CLL primary cells

CLL lymphocytes were (n=5) treated with 10 µM L14R8 for 2, 4, 6, 8, and 10 hours and cell death

was measured. PAC1a was used as a negative control. L14R8-induced apoptosis was measured by

Annexin V/PI staining method. Statistical analysis was performed using one-way ANOVA (*P<0.05).
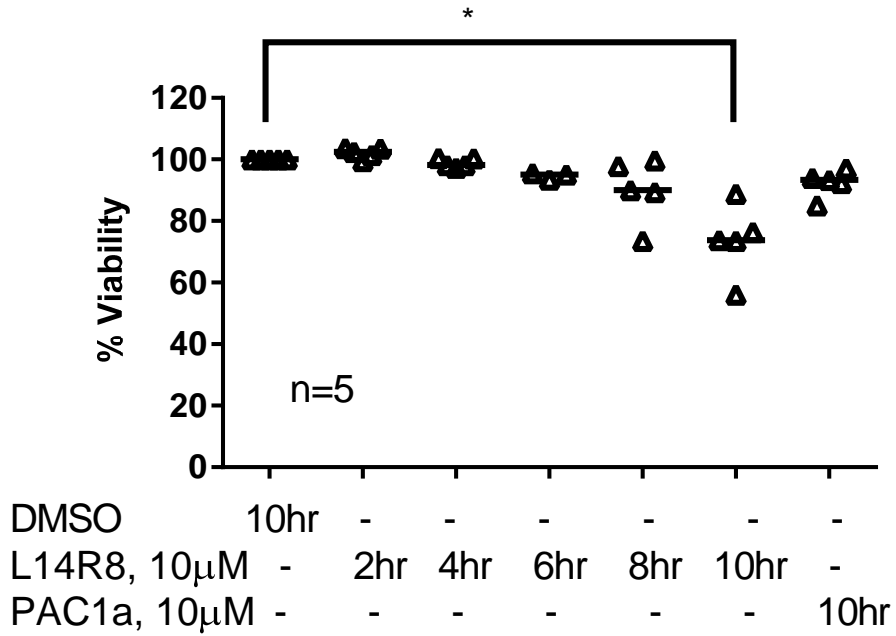
Table 7: Effect of L14R8 treatment on normal and malignant cells

Effect of L14R8 treatment on purified normal B cells (n=3), normal peripheral blood mononuclear cells (PBMCs) (n=3) and malignant CLL B cells (n=38). Cell cultures were treated with 10 µM L14R8 for 24 hours. % cell death was measured by Annexin V/PI staining method.

% Apoptosis			
Cell type	n	Median	Range
Normal B cells	3	31%	19-53%
Normal PBMCs	3	18%	16-28%
CLL cells	38	64%	18-90%

Figure 30: Impact of stroma on L14R8 induced apoptosis in CLL cells

CLL lymphocytes from 4 patients were cultured in presence or absence of stromal cells for 24 hours. These cultures were further treated with 5 and 10 μM L14R8 for 24 hours, and effect of stromal cells on L14R8-induced apoptosis was measured by Annexin V/PI staining method. Statistical analysis was performed using 2way ANOVA (n=4; *P<0.05; **P<0.01).
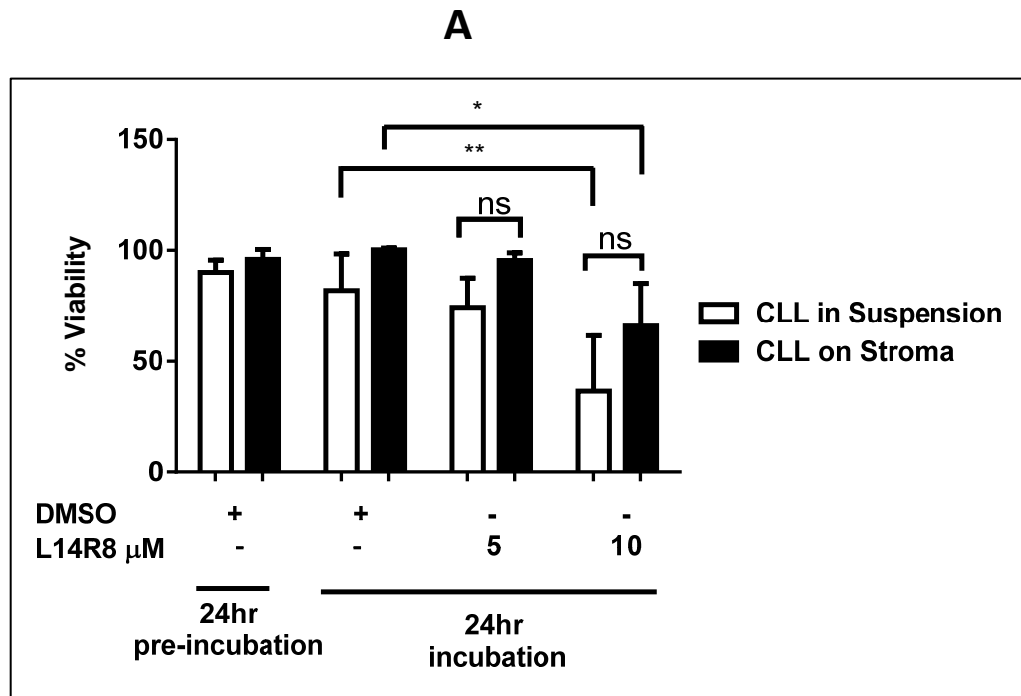
Figure 31: Impact of IL-6, IgM and CD40L on L14R8-induced apoptosis in CLL cells

Impact of microenvironment factors such as IL-6, IgM and CD40L on apoptotic action of L14R8 in CLL lymphocytes was measured. CLL cells from 11 patients were cultured in suspension with or without IL-6, IgM and CD40L for 48 hours and then treated with 10 μM L14R8 for 24 hours and % cell death was measured by Annexin V/PI staining method. Statistical analysis was performed using 2way ANOVA (n=11; ****P<0.0001).
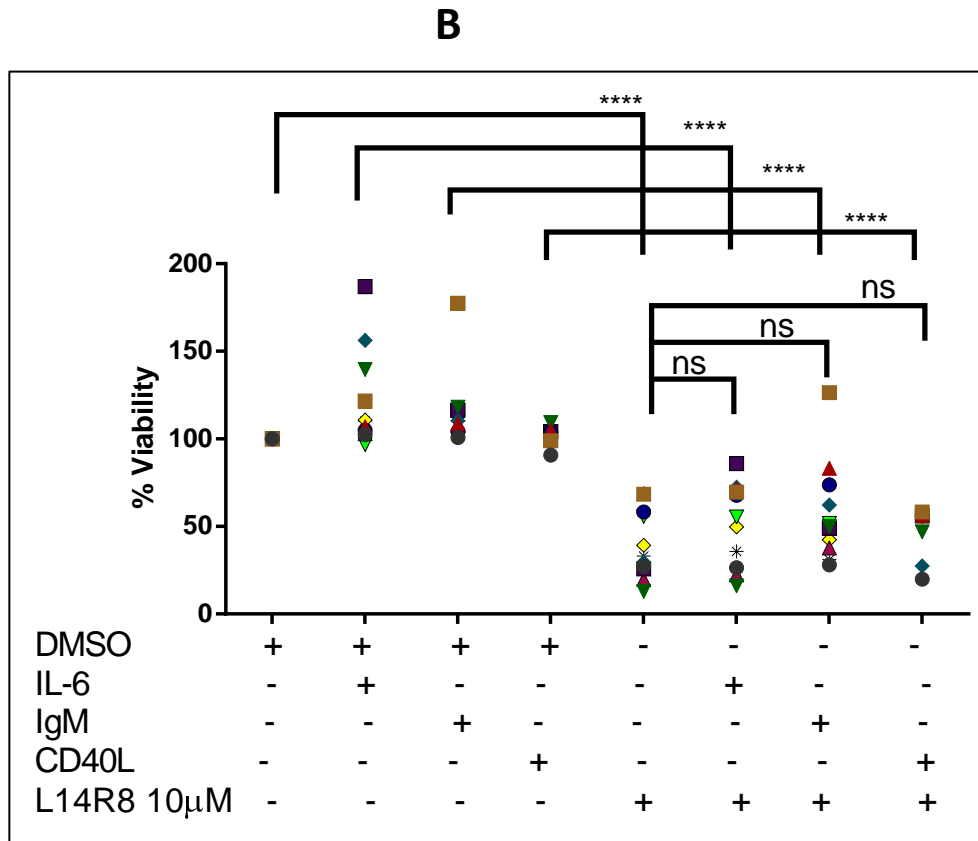
Figure 32: Effect of prognostic markers on L14R8-mediated cytotoxic response in CLL cells

% viability in CLL cells after 10 µM L14R8 treatment for 24 hours was compared with different Rai

stage (A), different cytogenetics (B), status of 17p deletion (C), status of IgVH mutation (D), status

of ZAP70 positivity (E), presence of prior fludarabine exposure (F), count of white blood cells (G),

and level of β2-microglobulin expression (H). Statistical analysis was performed using unpaired

student t test (B, D-G), One sample t test (hypothetical value 50; C), One-way ANOVA (**p<0.01; H).
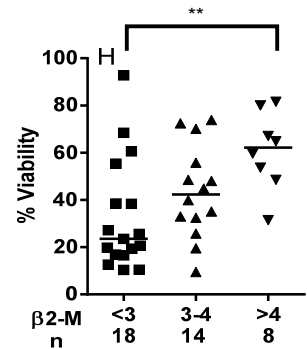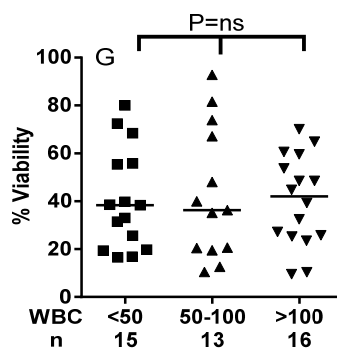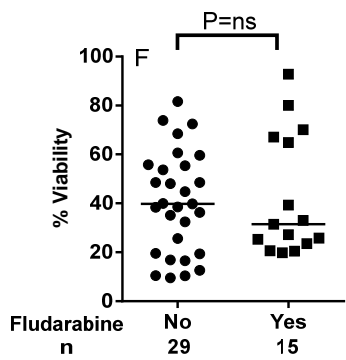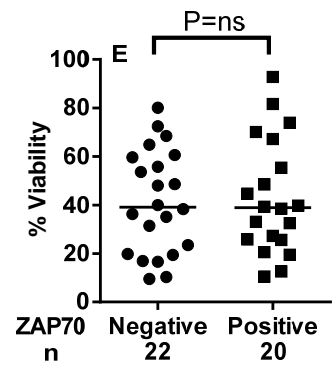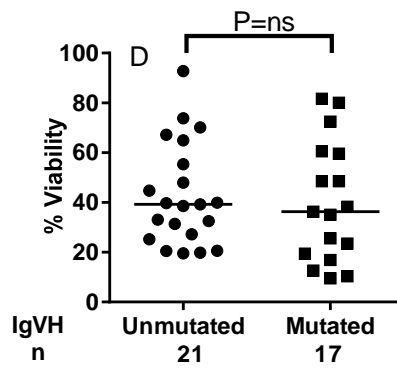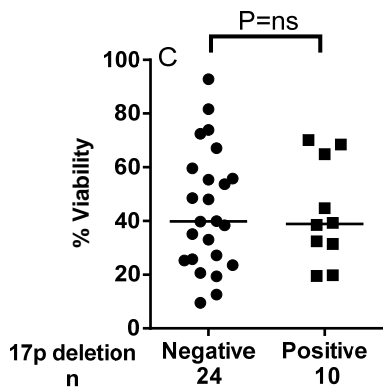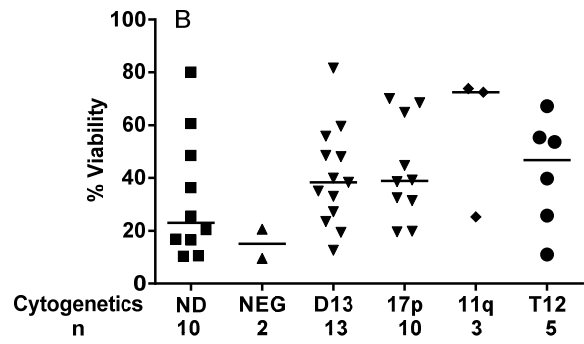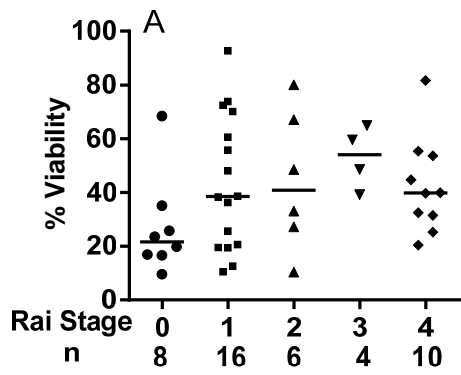
Aim 1.2: To determine the mechanism by which L14R8 induces apoptosis in in CLL
primary samples

4.5: L14R8 treatment promoted activation of procaspases in primary CLL cells

To test the effect of L14R8 on activation of target procaspases, protein expressions of initiator procaspases-8 and -9, and executioner procaspases-3 and -7, were analyzed after 24 hour treatment with 10 µM L14R8. Immunoblots of one representative patient data are shown in Figure 33A, 34A. L14R8 treatment activated caspase-3 and -7 by cleavage in CLL lymphocytes (Figure 33B and 33D respectively]. Procaspase-6 cleavage was not significantly different as compared to untreated control, indicating not a direct and significant involvement of caspase-6 in L14R8 action (Figure 33C). L14R8 treatment increased protein expression of both active caspase-9 (Figure 34A and 34B) and active caspase-8 (Figure 34A and 34C) indicating activation of both intrinsic and extrinsic pathways. Moreover, Parp protein cleavage hallmarks the activation of executioner caspases. Expressions of uncleaved Parp protein decreased and cleaved Parp protein increased with L14R8 treatment in CLL lymphocytes (Figure 35).

4.6: L14R8-induced apoptosis was not through caspase-8 cleavage

To test role of caspase-8 in L14R8-mediated downstream events of apoptosis, caspase-8 (-/-) jurkat cells were treated with L14R8 for 24 hours. L14R8 treatment induced apoptosis in WT ($p<0.0001$) and caspase-8 (-/-) ($p<0.0001$) cells significantly compared to respective controls, albeit at lower rate in caspase-8 (-/-) cells (Figure 36). Staurosporine treatment which is mediated by caspase-8 and was used as positive control, also induced cell death at same extent in caspase-8 WT or (-/-) cells. In contrast, FasL-induced cell death was only observed in WT cells for caspase 8. This data indicated that L14R8 was able to exert its effect even in the absence of caspase-8.

4.7: L14R8 induced apoptosis was procaspase-3 and -7 dependent

To test the involvement of executioner procaspases-3 and -7 in L14R8-mediated apoptosis, WT and caspase-3 and -7 (3/7) double knock out (-/-) MEFs were incubated with 5 µM L14R8 for 24 hours. L14R8 significantly decreased % viability in WT and caspase 3/7 (+/-) MEFs (p<0.0001) but not in caspase 3/7 -/- MEFs (p=ns) (Figure 37). These data demonstrated that presence of caspase 3/7 was critically required for L14R8-mediated apoptosis.

4.8: L14R8 induced apoptosis was bax/bak independent

It has been established that Bax and Bak proteins are required for mitochondria-mediated (intrinsic pathway) cell death. However, L14R8 should bypass this as it is targeting terminal proteins, procaspase-3 and -7. To rule out the possibility of factors involved upstream of executioner caspases in L14R8-mediated apoptosis, WT and Bax/Bak (-/-) double knockout MEFs were treated with L14R8 for 24 hours. L14R8 induced significant apoptosis in WT (p=0.0072) as well as Bax/Bak -/- (p=0.0345) MEFs (Figure 38). This is in contrast to staurosporine-mediated cell death, a positive control, which required Bax/Bak.

4.9: Temporal relationship between L14R8-mediated events of procaspase-3 activation and mitochondrial membrane permeabilization

To further evaluate the involvement of mitochondria in L14R8-mediated apoptosis, comparison between mitochondrial outer membrane potential (MOMP), cell survival and cleavage of procaspase-3 was carried out. MOMP was derived through geometric mean of TMRE stain (Figure 39A). Procaspase-3 cleavage was measured after immunoblot analyses of procaspase-3 and cleaved caspase-3 in CLL cells treated with 5, 10, 20 µM L14R8 for shorter times (2, 4, 6, and 10 hours) (Figure 39B). The protein density was measured using LI-COR Odyssey Infrared Imager, and quantitated (Figure 39B) for shorter incubations with L14R8 in CLL lymphocytes. The decrease in

fold changes of MOMP and procaspase-3 (R^2=0.78; p=0.01; n=21; data not shown), MOMP and viability (R^2=0.67; p<0.001; n=23; data not shown), and procaspases and viability (R^2=0.61; p<0.001; n=23; data not shown), correlated strongly and significantly indicating the temporal relation between executioner caspases activation and MOMP.

4.10: L14R8-mediated apoptosis of CLL cells was reversible and through intracellular Zn ion chelation

PAC-1 activated caspase-3 and caspase-7 through Zn ion chelation and removed Zn ion-mediated inhibition [139]. To test if L14R8-mediated apoptosis was Zn ion dependent in CLL, primary CLL cells were incubated with 10 µM L14R8 in presence or absence of 100 µM exogenous ZnSO4 for 24 hours. Exogenous Zn ion completely abrogated L14R8-mediated apoptosis (p=0.0063), while did not impact ABT-199 and staurosporine-induced apoptosis, whose actions are not zinc ion dependent. Treatment with Pac1a, a non-functional analogue did not induce apoptosis (Figure 40A).

Excess zinc ions may have chelated all the L14R8 molecules extra-cellularly in the medium, and hence the $ZnSO_4$-induced inhibition of apoptosis may not be due to its selective effect on procaspases. To exclude this possibility (i.e. extra-cellular chelation of Zn), exogenous zinc ions were added to CLL lymphocytes after the lymphocytes had been incubated with L14R8 for 5 hours to ensure L14R8 cell permeabilization. The time point of 5 hours was selected because L14R8 had induced apoptosis in CLL lymphocytes after as little as 6 hours of incubation (Figure 29). The zinc ions led to similar apoptosis rates when added simultaneously with L14R8 or when added after 5 hours of L14R8 incubation; in addition, the apoptosis rates were zinc ion concentration dependent regardless of when the zinc ions were added (Figure 40B).

4.11: L14R8-mediated apoptosis was reversible through pan-caspase inhibitors

To test if L14R8 induced apoptosis is reversed by caspase inhibition, pan-caspase inhibitors Z-VAD and Q-VD-OPH were used. % viability of CLL lymphocytes was significantly recovered from 15% in presence of L14R8 alone to 41% in the presence of Z-VAD (p<0.039) and to 65% in the presence of Q-VD-OPH (p<0.009) (Figure 41). Moreover, to further test if L14R8 induced apoptosis is reversible upon drug removal, after 5 hours of incubation with L14R8, CLL cells were washed of drug and were incubated for additional 18 hours. CLL lymphocytes washed after 5 hours of L14R8 incubation showed 96% viability, a significant recovery (p<0.0016) compared to CLL lymphocytes with continued L14R8 incubation which had only 21% viability (Figure 41), indicating the reversal of cytotoxicity of L14R8. These results demonstrated that L14R8-mediated Zinc ion chelation was reversible.

Figure 33: Effect of L14R8 treatment on activation of executioner procaspases

CLL lymphocytes were incubated in the presence or absence of 10 µM L14R8 for 24 hours. Protein expressions of executioner caspases were measured by western blot analysis as shown in (A). Caspases expressions from immunoblots were quantified using Odyssey Infrared Imager (LI-COR, Inc.) software for all patients analyzed and are shown in graphs; quantification of procaspase-3 and caspase-3 proteins (B) (n=9), quantification of procaspase-6 protein (C) (n=6), quantification of procaspase-7 and caspase-7 proteins (D) (n=11). Statistical analysis was performed using unpaired student t test (*p<0.05; **p<0.01; ***P<0.001).

B

Procaspase-3 Protein Expression

Caspase-3 Protein Expression

C

Procaspase-6 expression

D

Caspase-7 expression

Procaspase-7 expression
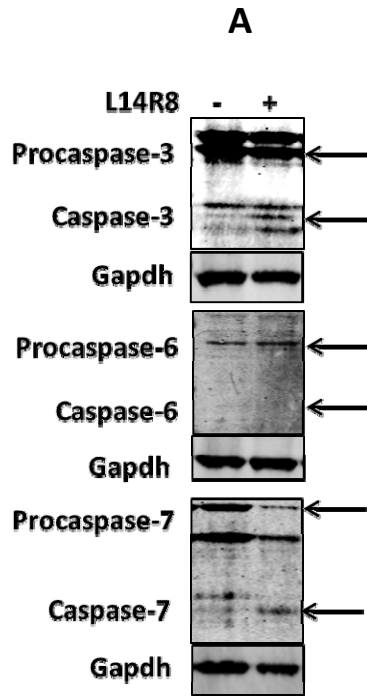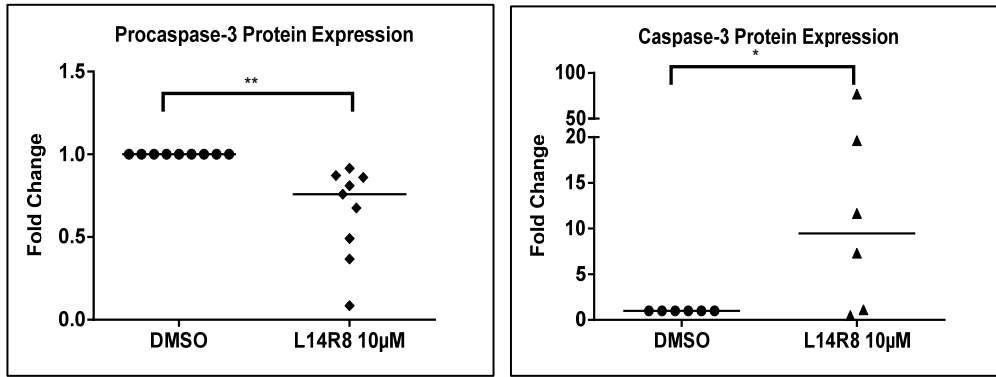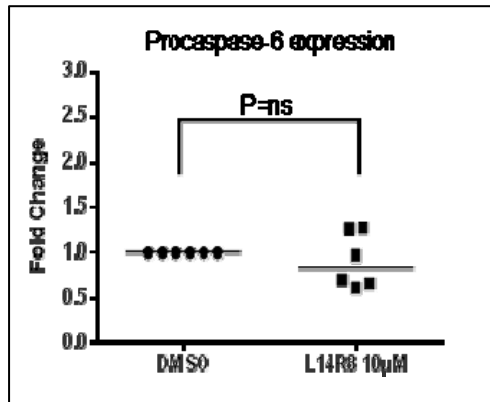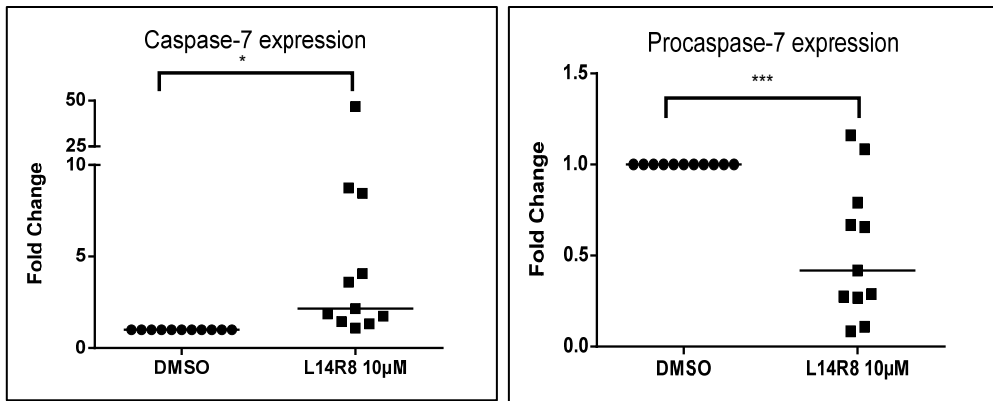
Figure 34: Effect of L14R8 treatment on activation of initiator procaspases

CLL lymphocytes were incubated in the presence or absence of 10 µM L14R8 for 24 hours. Protein expressions of initiator caspases were measured by western blot analysis as shown in (A). Caspases expressions from immunoblots were quantified using Odyssey Infrared Imager (LI-COR, Inc.) software for all patients (n=8) analyzed and are shown in graphs; quantification of procaspase-9 and caspase-9 protein expressions (B), quantification of procaspase-8 and caspase-8 protein expressions (C). Statistical analysis was performed using unpaired student t test (*p<0.05; **p<0.01; ***P<0.001).

A

B

Procaspase-9 expression

Caspase-9 expression

C

Procaspase-8 expression

Caspase-8 expression
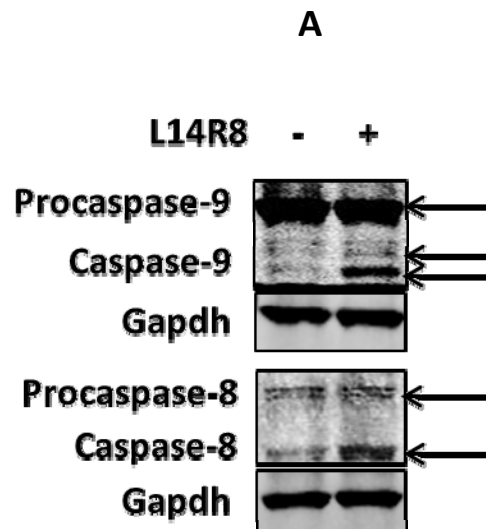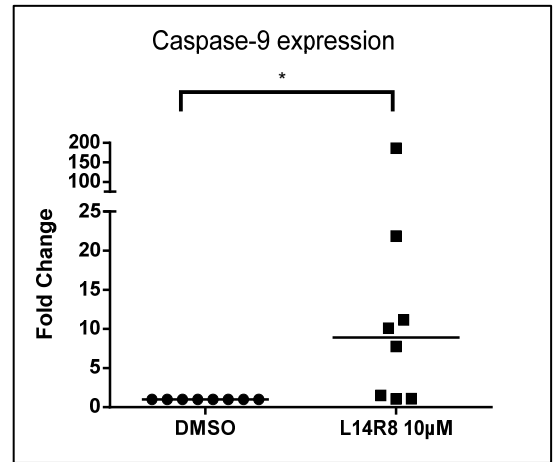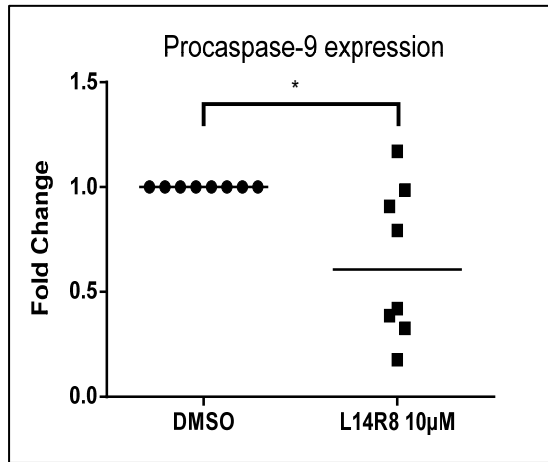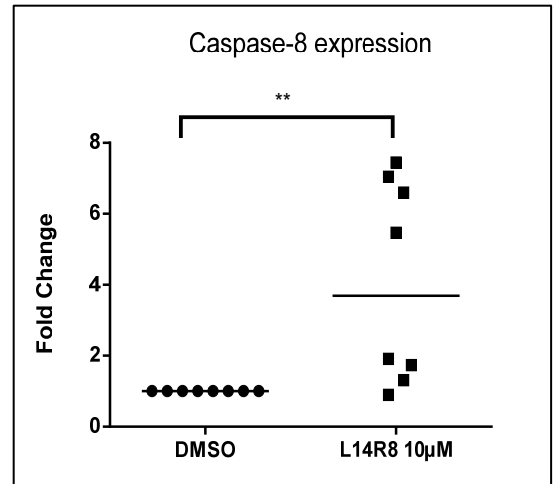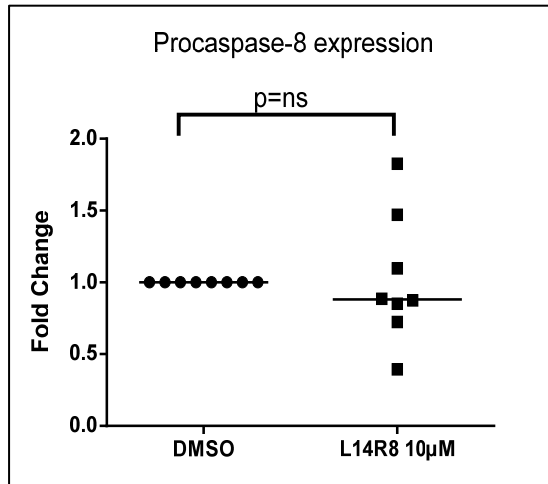
Figure 35: Effect of L14R8 treatment on Parp protein in CLL lymphocytes

CLL lymphocytes were incubated with 10 µM L14R8 for 24 hours and levels of uncleaved and cleaved Parp protein expressions were measured through western blot analysis (A). Uncleaved (B) and cleaved (C) Parp expressions were quantified using Odyssey Infrared Imager (LI-COR, Inc.) software for all patients (n=7) analyzed and shown in graphs. Statistical analysis was performed using unpaired student t test (****P<0.0001).
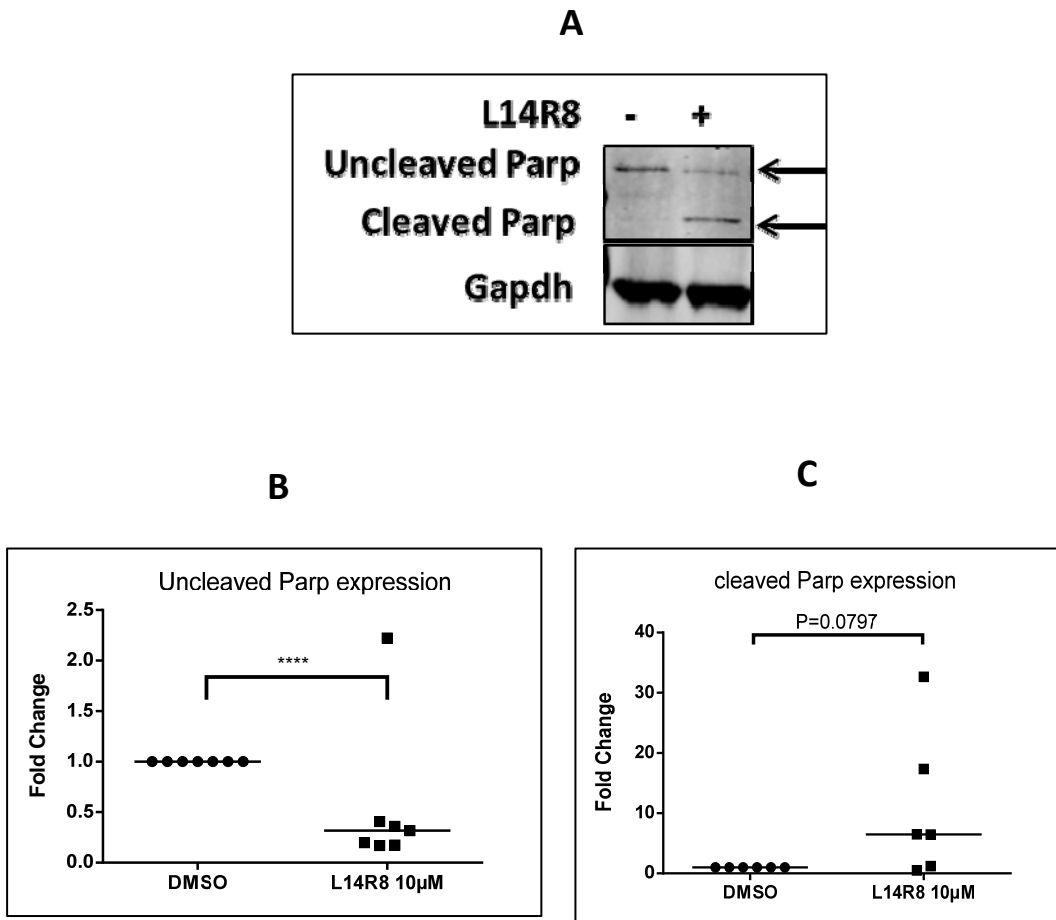
Figure 36: Role of caspase-8 in L14R8-mediated apoptosis

Wild type and caspase-8 knock out mouse embryonic fibroblasts (MEFs) were treated in the

presence or absence of 5 μM L14R8, 100 nM STS (staurosporine) and 100 ng/ml FasL (Fas Ligand)

for 24 hours. % cell death was measured by Annexin V/PI staining method. Statistical analysis was

performed using unpaired 2way ANOVA (****P<0.0001).
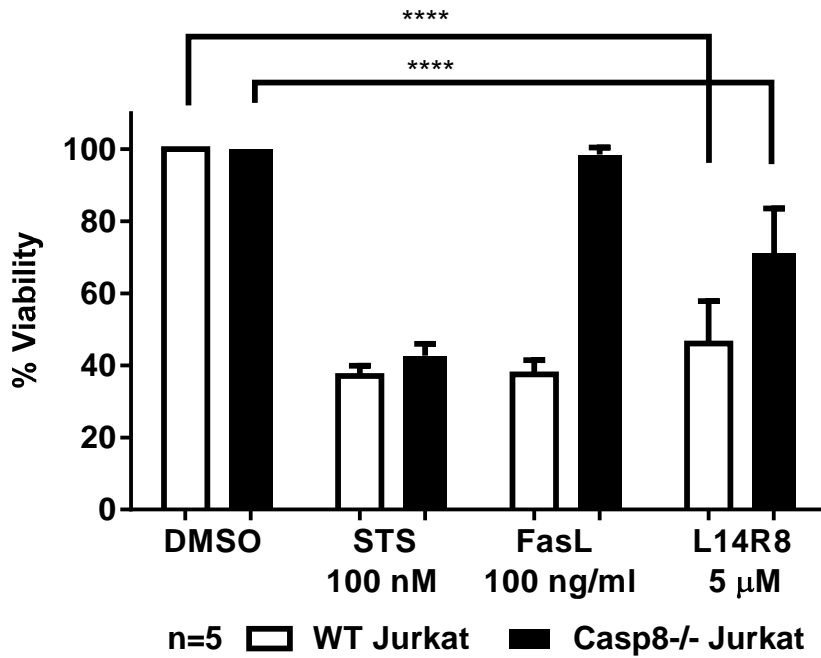
Figure 37: Role of caspase-3 and -7 in L14R8-mediated apoptosis

Wild type or caspase-3 and -7 single allele or caspase-3 and -7 double knock out mouse embryonic fibroblasts (MEF) were treated with either DMSO, 100 nM STS (staurosporine) or 5 µM L14R8 for 24 hours. % cell death was measured by Annexin V/PI staining method. Statistical analysis was performed using unpaired 2way ANOVA (****P<0.0001).
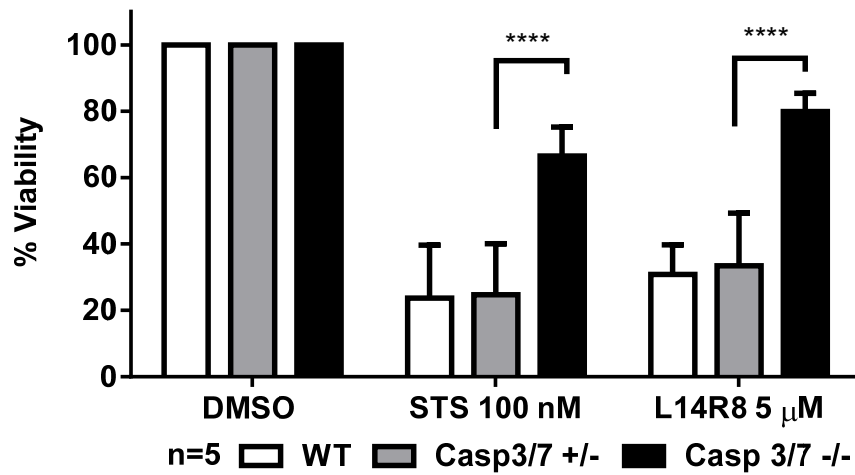
Figure 38: Role of Bax/Bak in L14R8-mediated apoptosis

Wild type or Bax/Bak double knock out mouse embryonic fibroblasts (MEFs) were treated with either 100 nM STS (staurosporine) or 2, 5 µM L14R8 for 24 hours. % cell death was measured by Annexin V/PI staining method. Statistical analysis was performed using unpaired 2way ANOVA (*p<0.05; **p<0.01; ****P<0.0001).
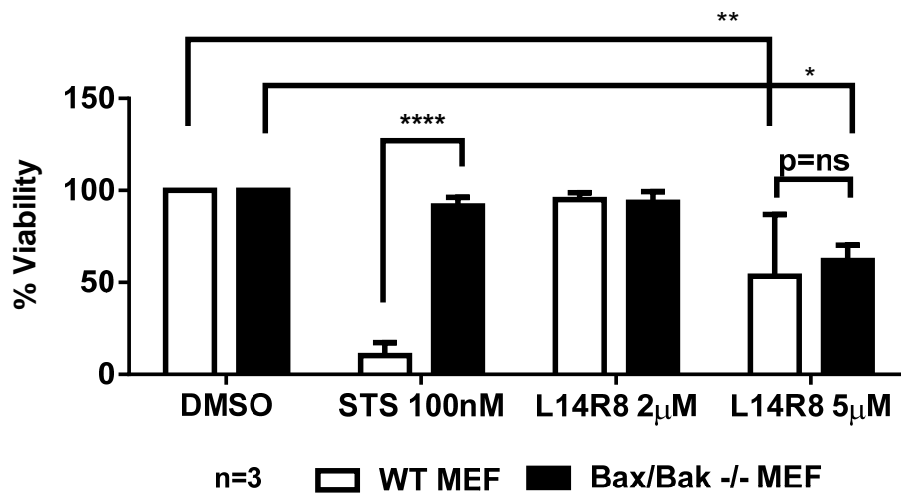
Figure 39: L14R8-induced mitochondrial membrane permeabilization and apoptosis

L14R8-induced mitochondrial outer membrane permeabilization (MOMP) was measured and compared with % viability and procaspase-3 cleavage in the same samples. CLL cells were treated with or without 10 µM L14R8 for 2, 4, 6, 8, and 10 hours. MOMP at 2, 6, and 10 hours was measured using TMRE stain and shown as histogram (A). Procaspase-3 cleavage induced by 5, 10, and 20 µM L14R8 at 2, 4, 6, and 10 hours were detected using western blot and quantitated (B). Temporal relation for MOMP, Procaspase-3 cleavage and cell death induced by L14R8 is presented (C). MOMP measured by TMRE stain was calculated as geometric mean. Fold changes in TMRE geometric mean, % viability and procaspase-3 cleavage induction in comparison to DMSO is plotted as graph. PAC1a was used as negative control.

A

	Control Stained
	L14R8 10μM
	PAC1a 10μM
	Control Unstained

B

C
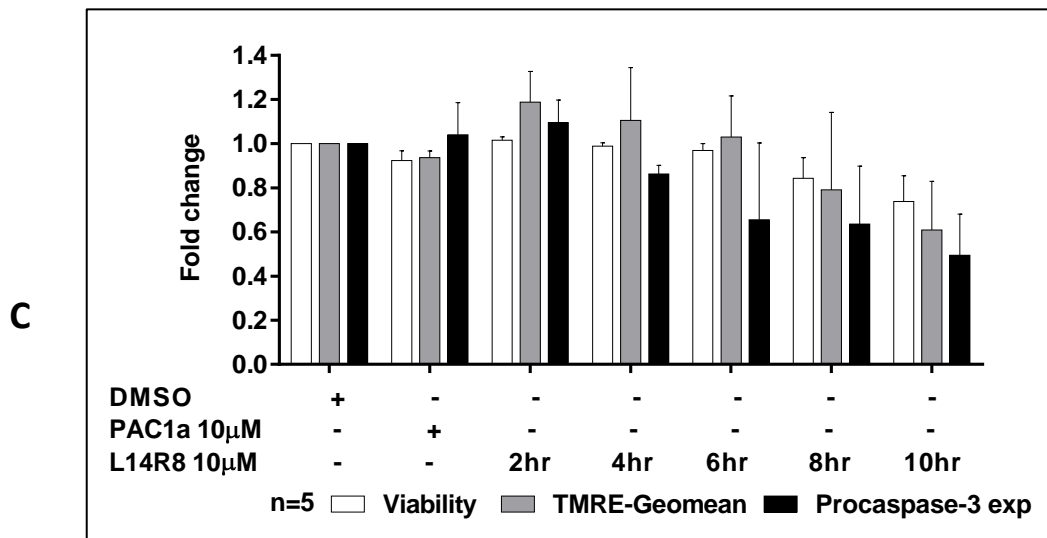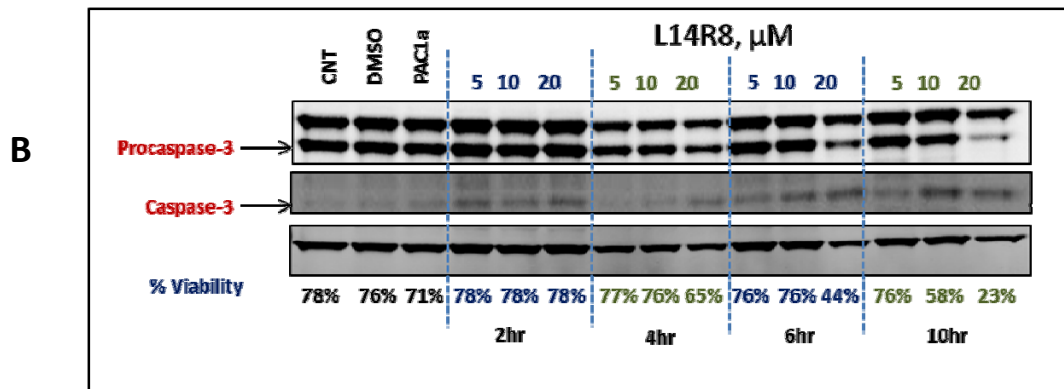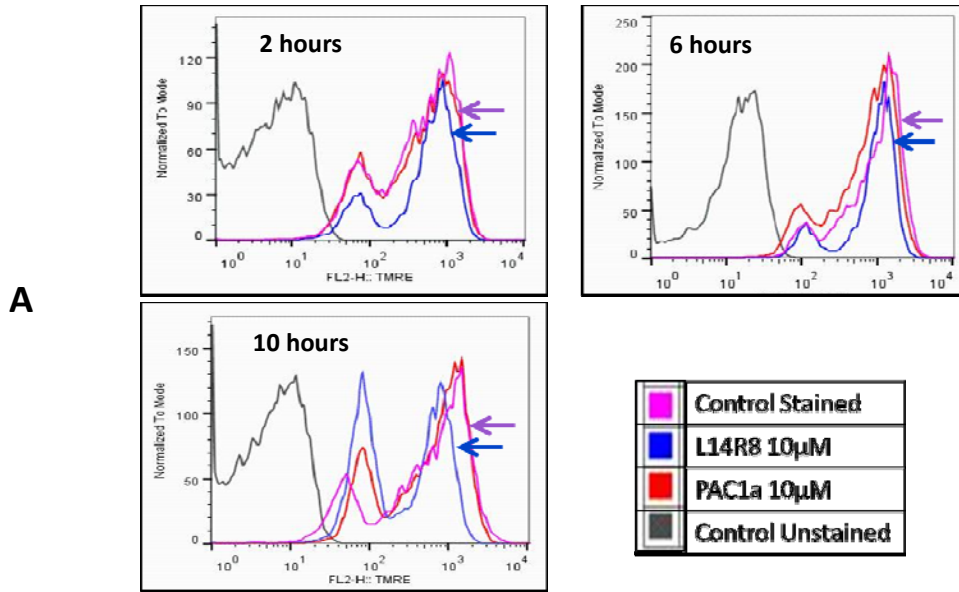
Figure 40: Action of L14R8 is through Zn ion chelation in procaspases

Effect of addition of exogenous Zn ion on L14R8-induced apoptosis was measured (A). CLL cells treated with 10 µM L14R8 in the presence or absence of 100 µM $ZnSO_4$ for24 hours. 1 nM ABT199 and 100 nM STS (staurosporine) were used as negative controls. Effect of different concentrations of exogenous $ZnSO_4$ on L14R8-mediated cell death was measured (B). 10, 50 and 100 µM $ZnSO_4$ were added to CLL cells with 10 µM L14R8 and cell death was measured at 24 hours (Solid line). Also 10, 50 and 100 µM $ZnSO_4$ were added to CLL cells 5 hours after addition of 10 µM L14R8 and cell death was measured at 24 hours of L14R8 incubation (dashed line). Statistical analysis was performed using unpaired One-way ANOVA (**$p<0.01$).

A

DMSO	+	-	-	-	-	-	-	-	-
PAC1a, 10μM	-	+	-	-	-	-	-	-	-
ZnSO$_4$, 100μM	-	-	+	-	+	-	+	-	+
ABT199 1nM	-	-	-	+	+	-	-	-	-
STS, 100 nM	-	-	-	-	-	+	+	-	-
L14R8, 10μM	-	-	-	-	-	-	-	+	+

B

DMSO	-	-	-	-	+
L14R8, μM	10	10	10	10	-
ZnSO$_4$, μM	-	10	50	100	-
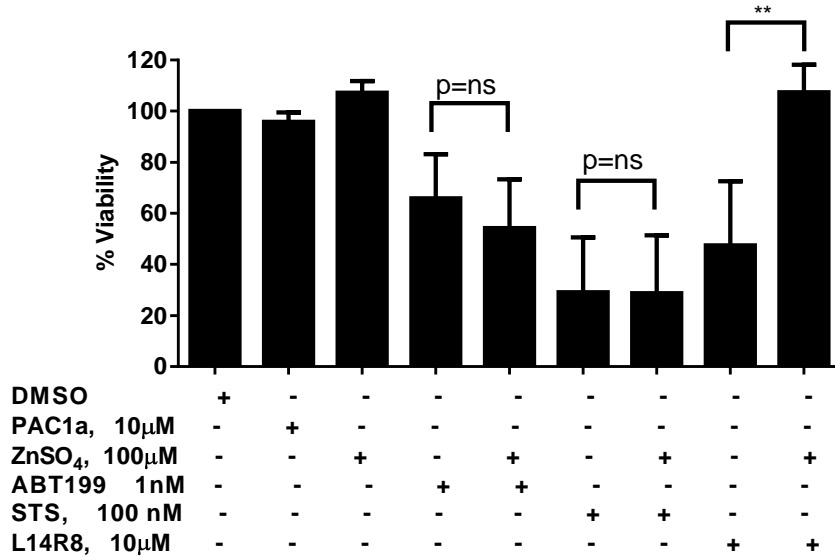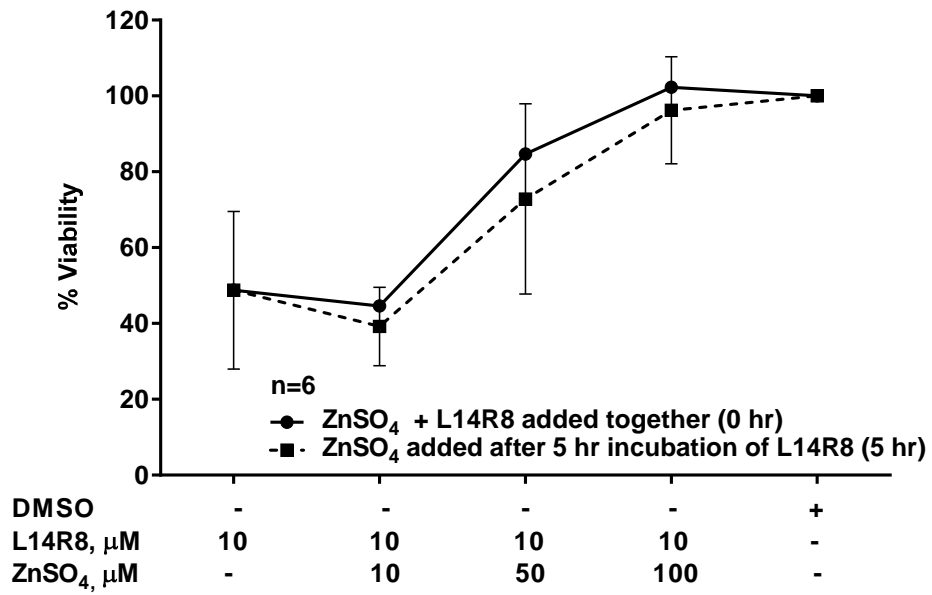
Figure 41: Effect of pancaspase inhibitors on L14R8-induced apoptosis

Effects of pancaspase inhibitors were analyzed on L14R8-mediated apoptosis. CLL lymphocytes were treated with L14R8 in the presence or absence of two pancaspase inhibitors, Z-VAD and Q-VD for 24 hours. Staurosporine (STS) was used as a control. Statistical analysis was performed using unpaired One-way ANOVA (*<0.05; **p<0.01).

DMSO	+	-	-	-	-	-	-	-	-
Z-VAD μM	-	50	-	-	50	-	-	50	-
Q-VD μM	-	-	50	-	-	50	-	-	50
STS nM	-	-	-	100	100	100	-	-	-
L14R8 μM	-	-	-	-	-	-	10	10	10
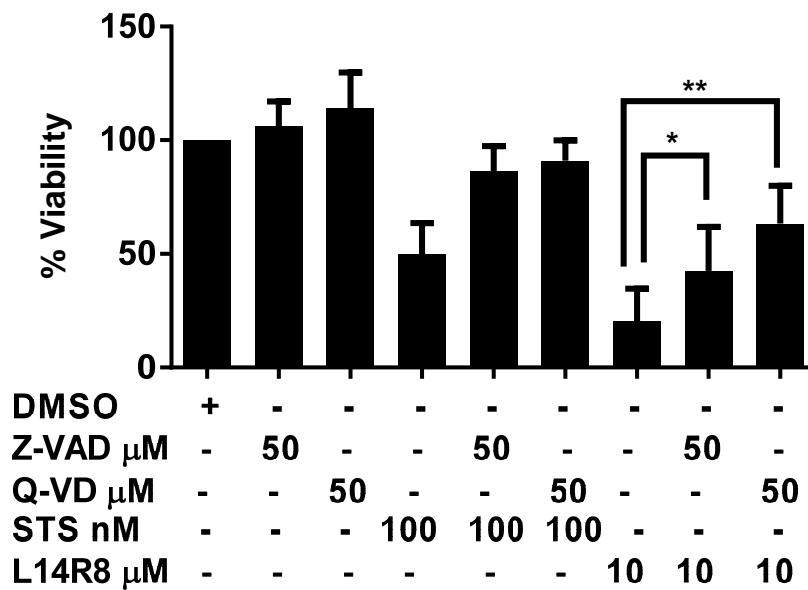
Figure 42: Reversibility of L14R8-induced apoptosis

Reversibility of L14R8-mediated apoptosis was tested. Two cohorts of CLL lymphocytes were treated with 10 μM L14R8. First set was incubated with L14R8 for 5 hours and then washed with PBS and continued incubation for 18 hours. The other set of CLL cells were continuously incubated with L14R8 for 23 hours. % viability was measured through Annexin/PI staining method and plotted. PAC1a was used as negative control. Statistical analysis was performed using unpaired One-way ANOVA (**p<0.01).
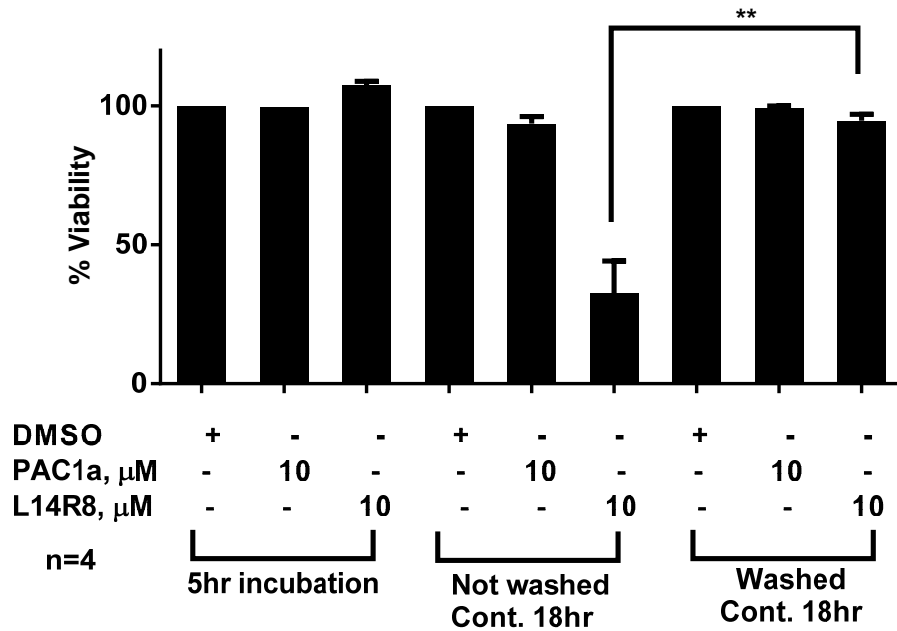
Aim 1.3: To test mechanism-based combinations of L14R8 in CLL primary samples

4.12: L14R8-mediated apoptosis was synergistic with Smac mimetics

Previously it was shown that IAPs inhibited caspases, and smac protein inhibited IAPs [149], [150], [151]. In order to further evaluate mechanism of L14R8, role of IAPs were studied. First, mRNA expressions of all IAPs in CLL lymphocytes were analyzed using real-time PCR microarray analysis. Our study demonstrated endogenous transcript expressions of NAIP1, cIAP1, cIAP2, XIAP and BRUCE in all samples analyzed (Table 8). We tested changes in these proteins expressions in the presence of L14R8 in CLL cells. Our study demonstrated that L14R8 treatment significantly increased Ciap1 (p=0.0097) and Survivin (p=0.0074) protein expressions in CLL cells (n=11; Figure 43A and 43B-C). However, Ciap2, Xiap, and Naip1 expressions were heterogeneous in CLL cells tested (Figure 43A and 43D-F). Interestingly, L14R8 treatment significantly increased expression of smac protein (p=0.0429) (Figure 45A-B).

Previously, it was demonstrated that CLL patient cells had higher expression of Ciap1 and Survivin proteins and lower expression of Smac proteins compared to PBMCs. Consistently, Ciap1 and Survivin protein expression was demonstrated to associate with worst outcome of treatment [38]. Our study demonstrated that L14R8 treatment increased Survivin and Ciap1 protein expression (Figure 43). Moreover, survivin protein expressions was significantly correlated with resistance to L14R8 treatment (Figure 44; p=0.026; r=0.73). Based on our observation, it was apparent that increased expressions of IAPs after L14R8 treatment prolong inhibition of caspases against L14R8 activity. Hence, we hypothesized that small molecule smac protein mimetic, Smac066, would inhibit IAPs and synergize L14R8-induced apoptosis. L14R8 and smac066 were tested for combination and showed significant decrease in % viability in combination at 2.5 and 5

μM concentration for patient # 102 (Figure 46A). Further, L14R8 and smac066 combination index

for other patient samples showed synergy (n=4; Figure 46B).

Table 8: transcript expressions of IAPs in CLL lymphocytes

Expression of IAPs in CLL lymphocytes were comprehensively analyzed using real time PCR array (microfluidic card) in 12 CLL patient samples. The presences of IAPs transcripts were analyzed in CLL lymphocytes in suspension cultures at 24 hours.

Function[152]	Gene name	Protein name	+ve in samples / total samples
Anti-apoptotic	BIRC1	NAIP1 (neuronal apoptosis inhibitory protein)	12/12
Anti-apoptotic	BIRC2	CIAP1 (cellular inhibitor of apoptosis 1)	12/12
Anti-apoptotic	BIRC3	CIAP2 (cellular inhibitor of apoptosis 2)	12/12
Anti-apoptotic	BIRC4	XIAP (X-chromosome binding IAP)	12/12
Anti-apoptotic	BIRC5	Survivin	10/12 (low expression)
Anti-apoptotic	BIRC6	Bruce (Apollon)	12/12
Anti-apoptotic	BIRC7	ML-IAP/KIAP (livin)	1/12
Anti-apoptotic	BIRC8	Ts-IAP/ILP2 (testis-specific IAP)	1/12

Figure 43: Effect of L14R8 treatment on expression of IAP proteins

CLL lymphocytes were incubated in suspension cultures in the presence or absence of 10 μM L14R8 for 24 hours. IAPs proteins expressions were measured through western blot and shown in the figure (A). IAP expressions were quantified using Odyssey Infrared Imager (LI-COR, Inc.) software for all patients analyzed and shown as graph; quantification of Survivin protein expression (B), quantification of Ciap1 protein expression (C), quantification of Xiap protein expression (D), quantification of Ciap2 protein expression (E), quantification of Naip1 protein expression (F). Statistical analysis was performed using unpaired student t test (**$p < 0.01$).

A

L14R8, 10 µM   −   +

Ciap1
Gapdh

Survivin
Gapdh

Naip1
Gapdh

Xiap
Gapdh

Ciap2
Gapdh

B

Survivin protein expression

**

Fold Change

DMSO   L14R8 10µM

C

Ciap1 protein expression

**

Fold Change

DMSO   L14R8 10µM

D

Xiap protein expression

p=ns

Fold Change

DMSO   L14R8 10µM

E

Ciap2 protein expression

p=ns

Fold Change

DMSO   L14R8 10µM

F

Naip1 protein expression

p=ns

Fold Change

DMSO   L14R8 10µM
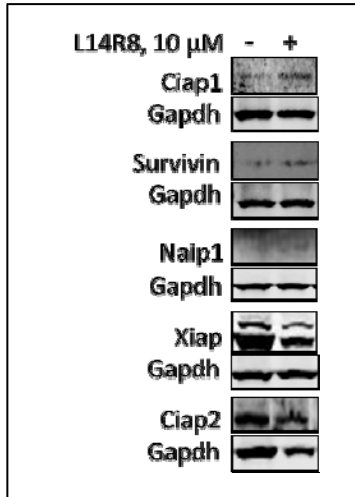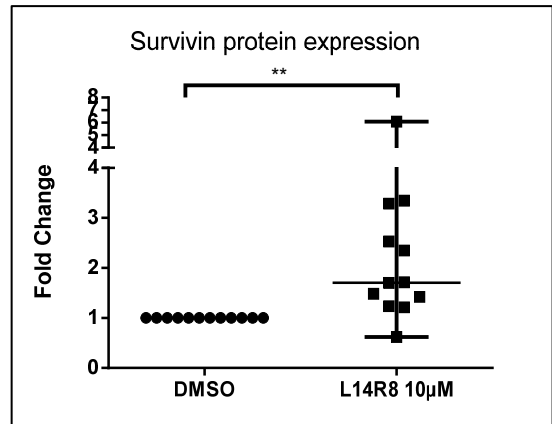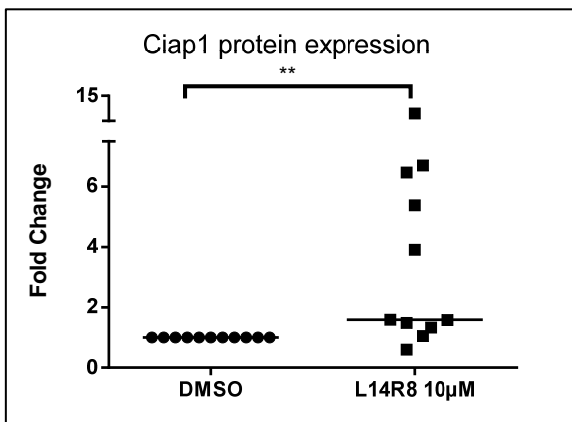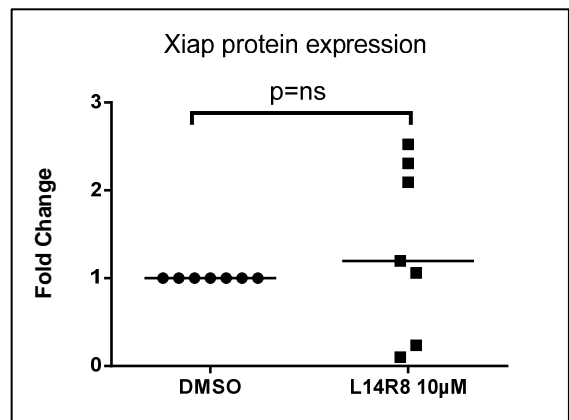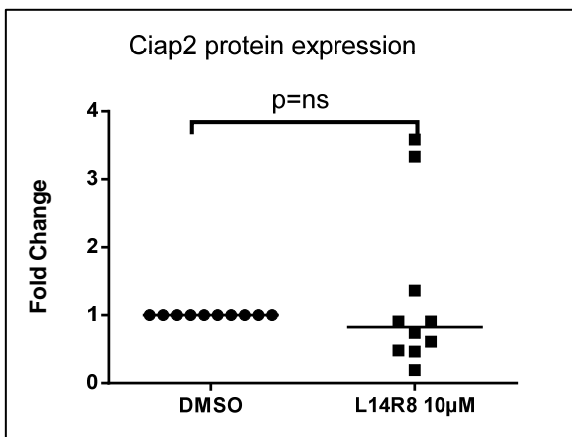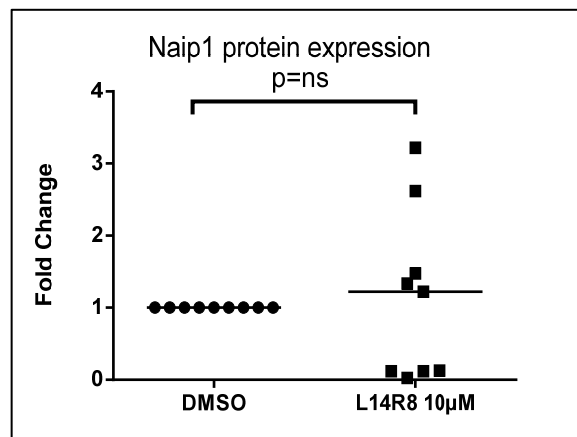
Figure 44: Correlation between L14R8 treatment-induced viability and survivin protein expression.

CLL cells were treated with 10 µM L14R8 for 24 hours and % viability was measured using Annexin V/PI staining method. Immunoblot analysis for survivin protein expressions in L14R8 treated and untreated CLL lymphocytes was carried out. Linear correlation between L14R8-induced viability and fold change in survivin protein expressions was analyzed. Statistical analysis was performed using linear correlation (*p<0.05).

P (two-tailed)	0.0258
P value summary	*
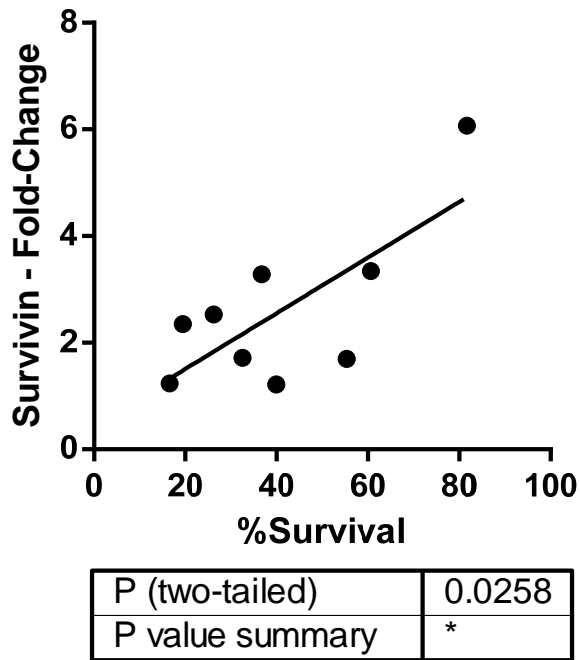
Figure 45: Effect of L14R8 treatment on expression of Smac protein

CLL lymphocytes were incubated in suspension cultures in the presence and absence of 10 µM

L14R8 for 24 hours. Smac protein expression was measured through western blot and shown in the

figure (A). Smac protein expressions were quantified using Odyssey Infrared Imager (LI-COR, Inc.)

software for all patients analyzed and shown as graph (B). Statistical analysis was performed using

unpaired student t test (*p<0.05).
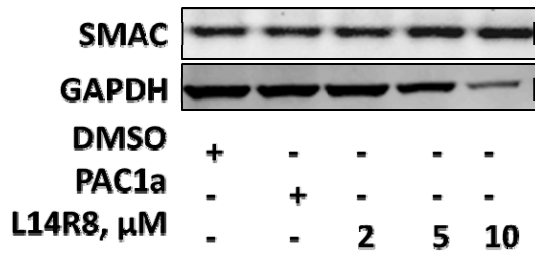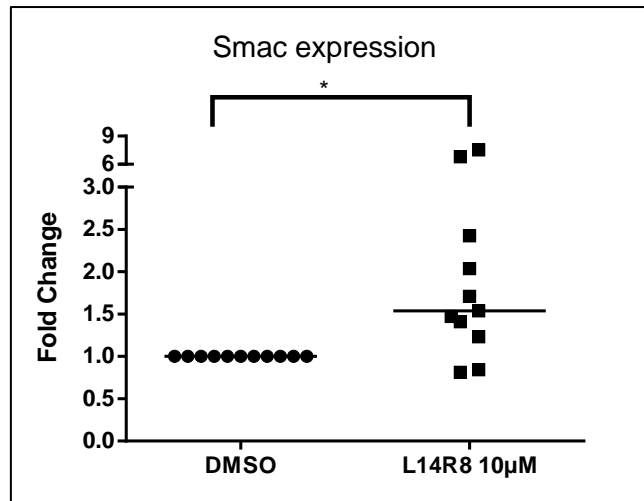
Figure 46: Mechanistic combination for synergy in L14R8-induced apoptosis

CLL lymphocytes were incubated in presence of either L14R8 or Smac066 or both agents with different concentrations for 24 hours and % viability was measured through Annexin/PI staining. Representative example of one patient data is presented (A). Combination index (CI) of L14R8 and Smac066 for all patients analyzed is presented (B); Values <1 indicate synergy. Combination index was measured through Calcusyn software.

A
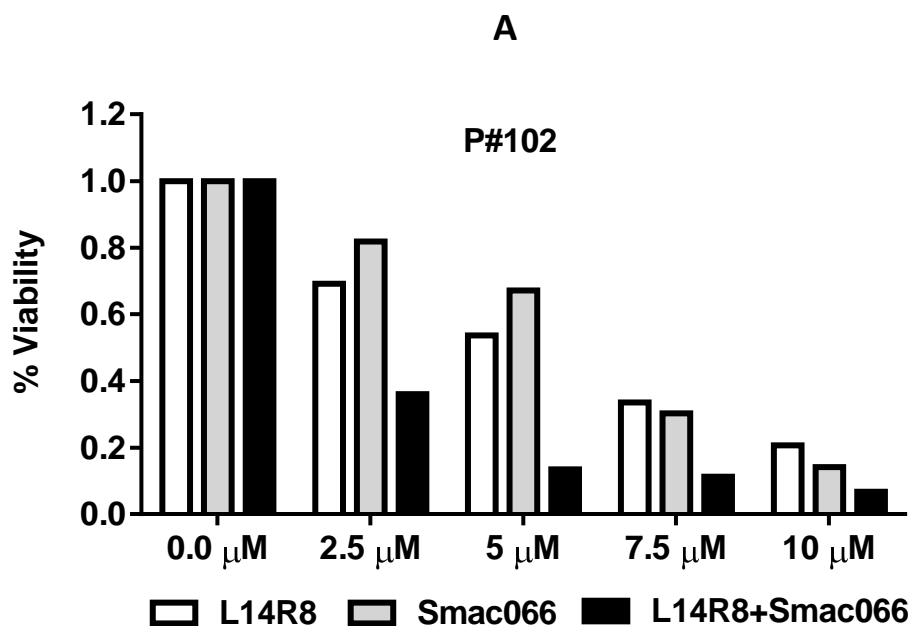
B

	CI values, µM for L14R8 and Smac066			
Pt No	**2.5+2.5**	**5+5**	**7.5+7.5**	**10+10**
P101	0.80	1.03	1.14	1.49
P102	0.74	0.75	1.02	1.02
P103	0.80	0.91	1.08	1.23
P105	1.50	1.02	1.00	0.71

CHAPTER 5: DISCUSSION

This study in CLL primary samples constitutes two parts of investigations. One involved the study of 93 apoptosis pathway transcripts, which were categorized in seven groups, in the presence of bone marrow stromal microenvironment in CLL lymphocytes. These groups also included essential Bcl-2 family members which are crucial regulators of apoptosis in CLL [153]. While several of these groups relate to apoptosis inducing proteins, many families or family members block apoptosis per se or apoptosis pathway. Second portion evaluated a novel compound that by-passes most of these apoptosis blocker proteins in the CLL lymphocytes that are known to relentlessly accumulate due to increased anti-apoptotic proteins.

5.1: CLL and stromal microenvironment interactions

The seven families of apoptotic pathways we studied are, TNF receptor superfamily (TNFRSF), Death domain (DED) and Caspase activation and recruitment domain (CARD), NF-kB, IAP, and caspase, and Bcl-2 families. Ample of research has been conducted to understand the role of microenvironment in the leukemic cells. Prior reports suggest that microenvironment aggravates the anti-apoptotic components in CLL cells but depletes the pro-apoptotic signaling that is essential to defense against the apoptosis resistance. The Involvement of BCR, TNFR2, MAPK/JNK, PI3K, NFKB and downstream Bcl-2, IAPs in the context of microenvironment has been documented. However, a comprehensive analysis of transcripts converging under multiple pathways in a model system of CLL and their concurrent regulation under microenvironmental conditions has never been investigated.

5.1.1: TNF receptor super family, DED and CARD family

Previously, studies showed that CLL cells receive survival signals mainly through surface B cell receptors and TNFRSF family receptors from diverse microenvironment. Moreover, it was also demonstrated that TNFRSF family members mediated signaling through interaction with DED family members [102]. In apoptosis pathway, TNFSF family receptor activate TNFR1 receptor signaling, which induces apoptosis through caspase-8 activation, and TNFR2 receptor signaling, which induces anti-apoptotic signaling pathway such as MAPK/JNK, NF-KB, and PI3K. Supporting these reports on TNFRSF receptor signaling, our study demonstrated that stromal cells activated TNFRSF signaling pathway and 5/22 hits were of this family (Table 4, Figure 18). These hits included Fas and TNF transcripts which is goes in line with previously findings that microenvironment factors activating CD40 receptor induced Fas protein expression in CLL cells [103,104]. In contrast, TNFRSF family members such as DR4 and DR6 were also increased which activate caspase-8 dependent pro-apoptotic signaling pathway. Another type of TNFRSF family receptor, TNFR2 receptor activation is known to induce activation of anti-apoptotic signaling; however, our data showed decrease in TNFR2 transcript expression under microenvironment. Along with TNF receptor signaling, stroma also induced transcript activation of associated adaptor pathway. Stroma induced 6/22 hits were of DED family as early stromal-interaction response. Stroma-induced increase in transcripts of adaptor molecules such as FADD and TRADD proteins suggested activation of downstream signaling pathway (Table 4, Figure 16). Again, for these families, we evaluated only transcript levels; however the protein levels should be analyzed as translation and post-translational modifications would induce complex interactions and functional importance in overall output.

Our study pointed out that analysis of apoptosis pathway transcripts in CLL cells under stromal co-culturing revealed a distinct pattern of changes dependent on incubation durations.

With that respect, it is interesting to note that stroma-induced changes in transcript levels of

TNFRSF and DED family pathway were only at short (1 day) duration of co-culturing and were not

detected at longer (3 days) time suggesting involvement of these pathways only transiently and

only at early stage of CLL-stromal interactions (Table 4, Figure 16, 18).

5.1.2: NF-kB, IAP and caspase families

NF-kB is a nuclear transfection factor composed of proteins from Rel family such as c-Rel,

RelA, RelB, p50 and p52. Inactive NF-kB protein is sequestered in cytoplasm by inhibitors such as

IkB family members, IkB-α, -β, -γ, and Bcl-3 [154]. Stimulators such as RANK (Receptor activator of

NF-kB), TNF, IL-1 and oxidative stress activate IKK kinase family members, which phosphorylate

members of IkB leading to liberation of cytoplasmic NF-kB followed by translocation to the nucleus

[155]. In the nucleus, NF-kB transcribes of pro-survival factors such as Bcl-2, Bcl-xL, and cFLIP, and

anti- survival factors such as FADD, and TNF [156]. Involvement of microenvironment induced

activation of NF-kB signaling pathway in CLL survival was evidenced previously and reported

increase of DNA binding of NF-kB transcription factor Rel A and its association with Bcl-2, Mcl-1 and

Bcl-xL transcription [44]. We did not see significant changes in transcripts of NF-kB family members

at shorter incubations. However, our study demonstrated strong decrease of IkB family members

IKBE and IKBZ at longer incubations indicating activation of NF-kB signaling. This may reflect the

activation NF-kB complex at later stage playing role in survival through activation of anti-apoptotic

proteins transcripts. However, it is to keep in mind that post translational modification of NF-kB

family members play important role in function of NF-kB singling pathway and this needs to be

evaluated to completely understand impact of NF-kB pathway in CLL microenvironment

interactions.

IAPs inhibit caspases and function as anti-apoptotic factors in CLL. NF-kB signaling pathway

is shown to induce IAPs expression [41]. Protein expressions of IAPs have been shown to increase

in CLL cells due to stromal microenvironment [92]. Overall, there was not a significant change in

transcript levels of IAPs at 1 and 3 days of co-culturing. However, there was a trend towards an

increase in some of the IAP members, for example cIAP1 had a median 1.45 fold augmentation

(p=0.15; Figure 15) under longer stromal co-culturing albeit this was not statistically significant. We

demonstrate significant decrease in the DIABLO/SMAC transcript at day 3 (Table 4). It was

previously demonstrated that smac protein inhibited IAP proteins function by binding with BIR

domain and prevented IAPs binding with caspases [149], [150], [151]. In vivo mouse study by Hui KK

demonstrated that physiological role of smac proteins in apoptosis by inhibition of IAPs (XIAP,

cIAP1 and cIAP2) activity on executioner caspase-3 [157]. These functional roles of smac support

the notion that decrease level of smac would enhance IAPs activity in CLL cells that are influenced

by stroma cells. Interestingly, this decrease in smac level was not significant on day 1 but was

statistically significant on day 3 which followed similar pattern as of Bcl-2 family anti-apoptotic

proteins (Figure 23).

5.1.3: Bcl-2 family anti-apoptotic proteins

CLL is a type of hematology malignancy which thrives due to defective apoptosis rather

than uncontrolled proliferation. Anti-apoptotic proteins of Bcl-2 family are major roadblocks in

apoptosis execution in CLL and their expression is abnormally increased compared to normal B-cells

[34,36,158]. As mentioned in introduction, critical functional importance in CLL cell survival and

resistance to apoptosis has been documented for all six Bcl-2 family anti-apoptotic proteins.

[159,160]. Consistence with prior studies, our present investigation demonstrated endogenous

expression of transcripts (Table 5 and Figure 20) as well as protein levels (Figure 23) of five of six

anti-apoptotic Bcl-2 family members. Microenvironment significantly induced changes in Mcl-1, Bcl-x_L, and Bcl-w protein expressions in CLL cells (Figure 23).

5.1.4: Bcl-2 family pro-apoptotic proteins

Pro-apoptotic Bcl-2 family proteins function as inhibitor of actions of anti-apoptotic proteins. Apoptosis induction in CLL cells has been previously documented and is detailed in the introduction section [77-79]. Among multi-domain Bcl-2 family pro-apoptotic members, analyzed using real time RT-PCR array, we demonstrated expression of Bax and Bak transcripts but not Bok in CLL patient lymphocytes (Table 5). Consistent with prior reports i.e. involvement of Bax and Bak in apoptosis, our reports showed stromal cells-induced survival did not change expression of Bax and Bak mRNA and protein in CLL cells.

BH3 only pro-apoptotic Bcl-2 family proteins function as activators or sensitizers of apoptosis [83], [84-87], [88]. There are nine members of this family. Our study demonstrated transcript and protein expression of six of nine BH3 only pro-apoptotic Bcl-2 family members (Table 5 and Figure 22 and 25). Among pro-apoptotic Bcl-2 family proteins, 1 day stromal coculturing slightly decreased expressions of Bik protein (P=0.047) and Puma protein (P=0.072) and slightly increased expression of Mcl-1 protein in CLL cells. However, there was no change in Bax transcript and protein (Figure 22 and 25) expression was observed under stromal co-culturing. This suggests that other post-translational modifications of Bax protein such as oligomerization might play role in apoptosis [161]. Shorter survivals of CLL patients were associated with single nucleotide polymorphism in Bax gene promoter region and low expression of Bax protein in CLL cells [33]. Our data demonstrated stroma induced decrease in pro-apoptotic proteins at shorter time points and induced increase in expression of anti-apoptotic proteins at longer time points partly explain interactions playing role in CLL survival in under stromal cell co-culturing.

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In general, with microenvironment, only Bik and Puma levels were reduced in CLL cells after a day of interaction. Overall, our data establish that anti-apoptotic members of Bcl-2 family play critical role in survival of CLL cells after coculturing with BMSC while both multidomain and BH3 only domain pro-apoptotic members play minor or supportive role.

5.1.5: Interactions of Bcl-2 family pro- and anti-apoptotic proteins and CLL survival

Recent review on interactions of Bcl-2 family proteins illustrated four models 'Direct activation', 'Displacement', 'Embedded together' and 'Unified' for BH3 only pro-apoptotic, multidomain pro-apoptotic, and anti-apoptotic Bcl-2 family proteins [162]. BH3 only pro-apoptotic proteins activate directly (as activator) and indirectly (as sensitizer) multidomain proteins and induce apoptosis. Anti-apoptotic proteins interact with multidomain proteins to interrupt apoptosis and provide survival signals. BH3 only pro-apoptotic proteins inhibit anti-apoptotic proteins function by binding with them or with multidomain pro-apoptotic proteins. The complex interactions among anti-apoptotic, BH3 only pro-apoptotic and multidomain pro-apoptotic proteins decide overall output of mitochondrial membrane permeabilization-induced apoptosis. Multi domain proteins oligomerize and permeabilize outer membrane of mitochondria, which releases cytochrome C and other components of inter membrane space and leads to apoptosis.

Binding of anti-apoptotic proteins and BH3 only pro-apoptotic proteins to Bax and Bak proteins depends on relative concentrations and interaction affinities of these proteins [162]. Puma is common activator protein and has high affinity binding with Bax/Bak proteins and induces Bax/Bak-dependent MOMP. Previously, it was shown that Bik worked as apoptosis sensitizer by binding with Bcl-2, Bcl-xL, Bcl-w anti-apoptotic proteins to inhibit their activities.

Our study showed that stromal microenvironment decreased expression of Puma and Bik transcripts at 12 hours and proteins at 24 hours. Moreover, stroma increased expressions of Mcl-1

anti-apoptotic protein (p=0.01; Figure 23) in CLL cells at 24 hours. Our data supports these observations that Puma and Bik might involve at early stage of stromal coculturing and interact with anti-apoptotic protein Mcl-1 to reduce apoptotic signaling. Also stroma decreased Bak transcript at 12 hours indicating the possible decrease of Bak proteins at very early stromal-CLL cells interactions, as we did not notice change at 24 hours. These suggest that stromal cells trended to minimize pro-apoptotic proteins expressions in order to allow survival advantage as early interaction response. The possible interaction model for Bcl-2 family proteins at early stromal interactions is shown as Figure 47 (Top circle). At longer incubations, stromal cells increased in all anti-apoptotic proteins (except Bfl-1), but did not induce a change in BH3 only and multidomain pro-apoptotic proteins suggesting that anti-apoptotic signals being predominant. It also suggested that stroma induced CLL survival advantage by increasing anti-apoptotic proteins rather than by suppressing pro-apoptotic proteins with continued incubations. Figure 47 (bottom circle) illustrates model for these Bcl-2 proteins interactions for longer duration of CLL-stromal interactions.

Figure 47: Model for changes in Bcl-2 family proteins in CLL lymphocytes at short (top circle, 12hr-1 day) or long (bottom circle, 3 days) co-culturing with bone-marrow stromal cells
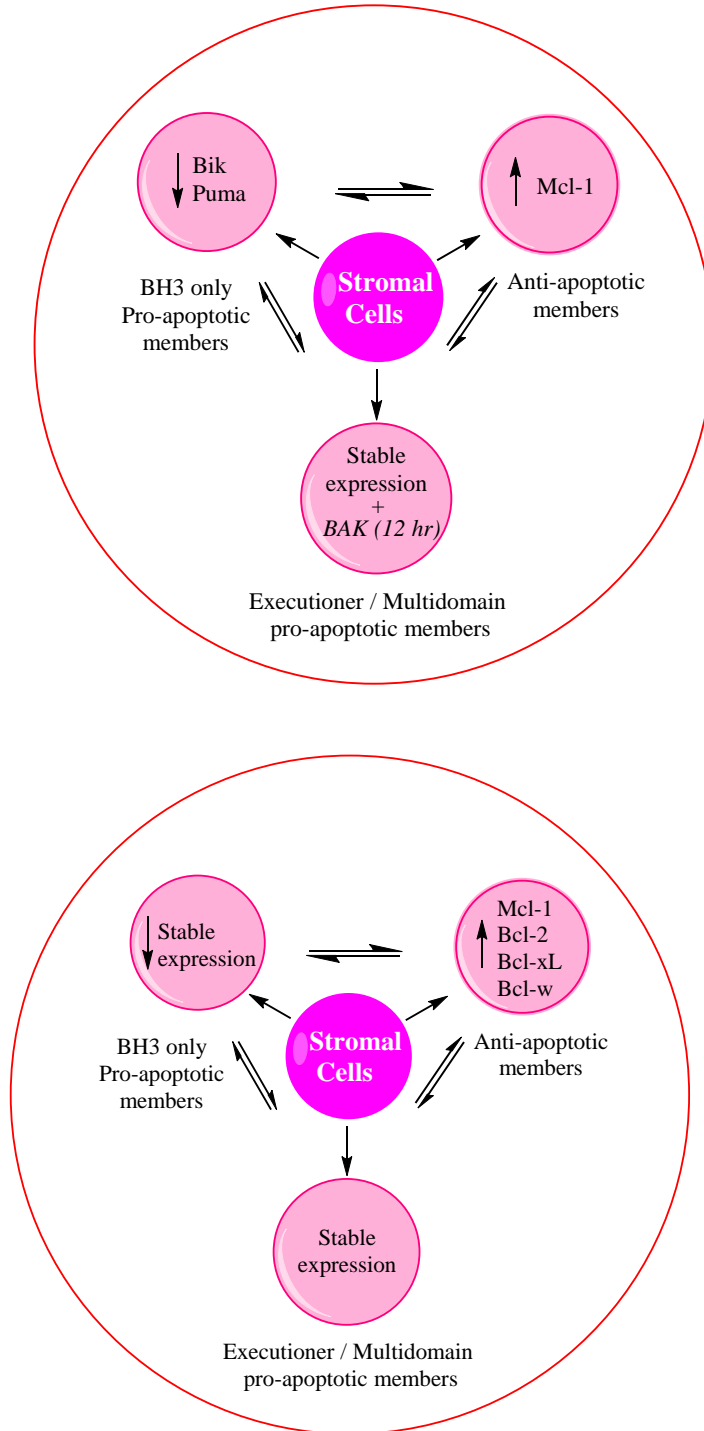
5.1.6: Spontaneous and drug-induced apoptosis and Bcl-2 family members

CLL cells have high expression of anti-apoptotic proteins while they are in peripheral blood. When CLL cell are present in bone marrow and lymph node microenvironment they obtained survival signals and increase apoptotic resistance and drug-induced resistance [163]. Several members of anti-apoptotic Bcl-2 family have been identified in CLL that result in endogenous or drug-induced apoptotic resistance. For example, Bfl-1 has been demonstrated to be involved in drug-induced resistance. Consistence with these observations, we had increased expression of BFL-1 transcripts under bone marrow stromal microenvironment. It has been evidenced that stroma decreased apoptotic priming in CLL cells [164], and Mcl-1 and Bcl-2 are involved in angiogenic switch under stromal environment [165]. In CLL cells, Mcl-1 was induced in follicular dendritic environment and Bcl-x_L was induced in fibroblast cells expressing CD40 receptors [50,166]. Studies have shown that many chemotherapeutic agents induced apoptosis in CLL cells in suspension cultures but resistance to apoptosis is developed under stromal coculturing [147]. Role of anti-apoptotic proteins have been documented in these scenarios and hence, attempts have been made to find small molecule inhibitors for these proteins to find therapies. Some of them are gossypol and its analogue, AT-101, ABT-737 and obatoclax, which specifically target anti-apoptotic Bcl-2 family proteins, shown to induce apoptosis in CLL cells even in presence of microenvironment [37]. This signifies our report of increase in anti-apoptotic proteins expression under stromal microenvironment.

5.1.7: Duration of co-culturing

Duration of the co-culturing is another factor of the study to be considered. Longer incubations possibly impart many variables and need to be considered in making overall conclusions. Longer durations, such as 7 days of co-culturing, demonstrated increase in expression

of Mcl-1, Bcl-2, and Bcl-x$_L$ proteins in CLL cells [44]. However, long durations of incubations have resulted in development of dendritic nurse-like cells and hence these long duration possibly involve survival signals from nurse like cells and development of resistance. With this respect, we had purposefully selected shorter co-culturing that has maximum of 3 days so that we could mimic and predict effects of bone marrow stromal microenvironment. During short incubations, at 1 day co-culturing, transcripts of both pro and anti-apoptotic proteins increased, however, only few were translated as increase in protein expressions. (Figure 20-25). Stroma induced early response of protein expression included decrease in pro-apoptotic 2/8 members, and stable expression of 5/8 members. Stroma induced late response of protein expression increased anti-apoptotic 4/5 members.

5.1.8: Stress vs. Survival: A decision to make?

Our study provided transcript expressions pattern at 2 different time points. 1 day stromal co-incubation time point (Figure 9A) was selected to represent onset of viability advantage and 72 hours (Figure 9C) stromal co-incubation time point was selected to represent complete viability advantage. As expected, we had observed 2 distinct pattern of gene expression on both days. Shorter duration of stromal co-culturing demonstrated increase in transcripts of both pro- and anti-apoptotic members i.e. Bcl-2 pro-apoptotic family, Bcl-2 anti-apoptotic family, DED family and TNFRSF family members in CLL cells; while longer duration of incubation showed decrease in transcripts of pro-apoptotic family members of NF-kB family, and transcripts of pro-apoptotic DIABLO/SMAC, and also demonstrated increase in proteins of anti-apoptotic Bcl-2 family.

Previously Lagneaux et al. demonstrated that normal cells incubated with stromal cells induced apoptosis, while CLL cells incubated with stromal cells induced survival. This finding indicates that stroma provided pro-apoptotic signals to normal B cells. It is possible that stroma

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also provided pro-apoptotic signals to CLL cells along with CLL cells. CD40 activation induced pro-apoptotic Fas receptors expression in CLL cells but NF-kB survival signaling dominated [104]. Our study also demonstrated that early phase of interaction of stromal cells induced expression of both pro and anti-apoptotic signals but only anti-apoptotic transcripts translated to proteins and dominated with further interactions in CLL cells (Table 4; Figure 20-25). Hence, it can be extrapolated that that stromal CLL cells interactions induced both the assembly of apoptosis pathway i.e. pro and anti- apoptotic members. However, altered CLL cells biology take advantage of neighbors and make decision of survival against stress.

5.1.9: Quality of results of Real time PCR microfluidic array

One may have concern about the specificity and significance of the results due to increase of both pro- and anti-apoptotic transcript expression at short stromal co-culturing but only increase of anti-apoptotic protein expression at longer co-culturing durations. We have confirmed our results through cross referencing and using control experiments. Below are three reasons through which we support quality of array results.

First, uridine incorporation showed that the global RNA synthesis rate of CLL increased in the presence of stroma (Figure 11). This reflects total RNA synthesis which includes mRNA, rRNA & tRNA synthesis. These are needed both for mRNA transcription, and protein synthesis. Because stroma activates CLL cells, increases in RNA and protein synthesis are expected. This experiment gave global view of RNA synthetic capacity of CLL cells and influence of stroma.

Second, both pro- & anti-apoptotic members' mRNA expressions were changed. Hence, to confirm the specificity of the array data we analyzed BIM mRNA expressions using TaqMan real time RT-PCR assays and individual real time PCR assay. The results correlated the mRNA expression data of real time PCR assays to that obtained from array data. We observed the significant and

strong correlation indicating the reliability of the data (Figure 8). Moreover, with the increase in duration of incubation, we observe that not all of the mRNAs which had higher expression at short durations are translated to proteins. We find only anti-apoptotic proteins increased at day 3 (except Bfl-1) in the presence of stroma which reflects the longer incubation viabilities (day 3-6) (Figure 9, 20).

Third, 1 day stromal cells incubation provided only 8% survival advantage compared to CLL cells in suspension; while 3 days stromal incubation provided 21% survival advantage to CLL cells (Figure 9). The difference of viability was predominant on day 3 which correlated with anti-apoptotic pattern. In addition, the early incubations also reflect exogenous stress signals to CLL cells due to *in vitro* coculturing. We also checked that at 1 day coculturing, among pro-apoptotic mRNAs only Bim, Puma & Bad are increased, while Bax and Bak mRNAs were unchanged and among anti-apoptotic mRNA only Bcl2A1 and Bcl-XL were increased, reflecting the biology of CLL-stromal interactions.

5.2: L14R8 treatment in CLL

Deregulation of apoptosis pathways in CLL contributes not only to disease pathogenesis but also to the development of resistance to cytotoxic agents. With respect to therapeutic stand point, B-cell Receptor kinases, Bcl-2 family anti-apoptotic proteins and IAP family proteins have emerged as potential targets in CLL. Despite encouraging clinical activities, the efficacy of these agents indeed depends upon the target protein levels in malignant cells followed by signaling of the downstream pathway. Here we are presenting a new approach of targeting CLL cells that is independent of pro-survival proteins or upstream regulators, but depends directly on manipulating the downstream caspases, a family of proteases that act in concert to execute apoptosis.

5.2.1: Apoptosis, prognostic markers, and microenvironment

The present study demonstrated that L14R8, a small molecule activator of procaspase, resulted in primary CLL cell death. This procaspase activation approach is used first time for CLL malignant lymphocytes. L14R8 was one of the potent hit in screening of 837 analogues of PAC-1 [140]. Preliminary data indicated that L14R8 induced cell death in lymphoma cell line [140]. Our investigations extend these preliminary data as we demonstrate utility of L14R8 in several patient samples. As expected due to the role of L14R8 on terminal caspases in the cell death pathway, the drug induced death in CLL cells irrespective of the status of prognostic factors, cytogenetic aberrations, prior fludarabine exposure, and IgVH gene mutation status. The only factor that impacted L14R8-mediated cell death was expression level of β2-microglobulin on CLL cells (Figure 32). We have not evaluated mechanism of β2-microglobulin protein and action of L14R8.

L14R8-mediated apoptosis of CLL cells was also observed in CLL cells that are in protective microenvironment such as NKTert co-cultures, presence of IL-6, CD40L, and IgM stimulation.

Consistent with the survival benefit of these factors, our data revealed slightly lower rate of L14R8-induced apoptosis in presence of microenvironment (Figure 31 and 32). Stromal microenvironment induced increased anti-apoptotic Bcl-2 family members [148] and anti-caspases IAPs expressions in CLL [92] providing survival advantage and drug resistance to CLL cells [37,94], which might explain decreased rate of L14R8-induced apoptosis in presence of stromal cells. However, L14R8-induced significant CLL cell death in the presence and absence of microenvironment. Previously Boldingh et al., showed epidermal growth factor decreased approximately 25% of cell death induced by PAC-1 through decrease in caspase-3 activation in PC12 neuron cells [167].

5.2.2: Executioner caspases activation

Selectivity of procaspase activating compounds towards cancer cells depends on protein expression levels of executioner procaspases. Different solid tumor types such as melanomas [168], neuroblastomas [169], lymphomas [170], and colon [171] and liquid cancers like acute myelogenous leukemia [172] showed high expressions of procaspase-3 compared to respective normal healthy cells. Hence, studies of PAC-1 application in dog lymphomas [173] and mice renal cancers [138] had selection advantage of high procaspase expression. Our study showed that L14R8 selectively induced apoptosis in CLL cells compared to PBMCs from healthy donors (Table 7). This specificity of L14R8 compound towards CLL patients' B-lymphocytes compared to normal B cells might be due to following reasons. First, CLL cells showed expression of executioner procaspase-3, and -7. Second, procaspase-3 and procaspase-7 were activated through zinc ion chelation through PAC-1 [139] and L14R8. Third, on the comparison basis, CLL cells had higher expression of procaspase-7 and -3 compared to PBMCs (Figure 33). Finally, CLL cells possessed abnormal apoptotic machinery compared to normal B-lymphocytes.

Initiator procaspases-8 and -9 are in monomer forms as zymogens and get activated by dimerization followed by further reinforcement through cleavage and maturation through catalytic regions. In contrast to initiator procaspases, effector or executioner procaspases (-3, -6, and -7) are in dimeric form as zymogens and get activated through initiator caspases and through activated effectors [136] [Reviewed]. Effector or executioner caspases have another level of regulation. Zinc previously demonstrated co-localizing with procaspases. Moreover, Peterson et al. clearly demonstrated that zinc ion inhibited procaspase-3 activation and removal of zinc ion activated executioner caspases [139]. In our study, loss of caspase-8 (Figure 36) or double knockout of Bak and Bax (Figure 38) did not abrogate L14R8-induced apoptosis. Whereas, knockdown of procaspase 3/7 (Figure 37) influenced L14R8-induced apoptosis supporting that the primary action is on and through procaspase-3 and -7. Reversal of L14R8 cytotoxicity with exogenous zinc ion (figure 40) cemented mechanism of action of L14R8, which is through zinc chelation and executioner procaspase activation. Figure 48 illustrates proposed L14R8 mechanisms in apoptotic pathways.

Figure 48: Illustration of proposed mechanisms of L14R8 in apoptotic pathways

Activation of extrinsic and intrinsic apoptotic pathways result in activation of executioner caspases (procaspase-3 and -7). L14R8 directly remove Zinc ion-mediated inhibition of executioner caspases. Activated caspase-3 and -7 initiate CLL cell death.
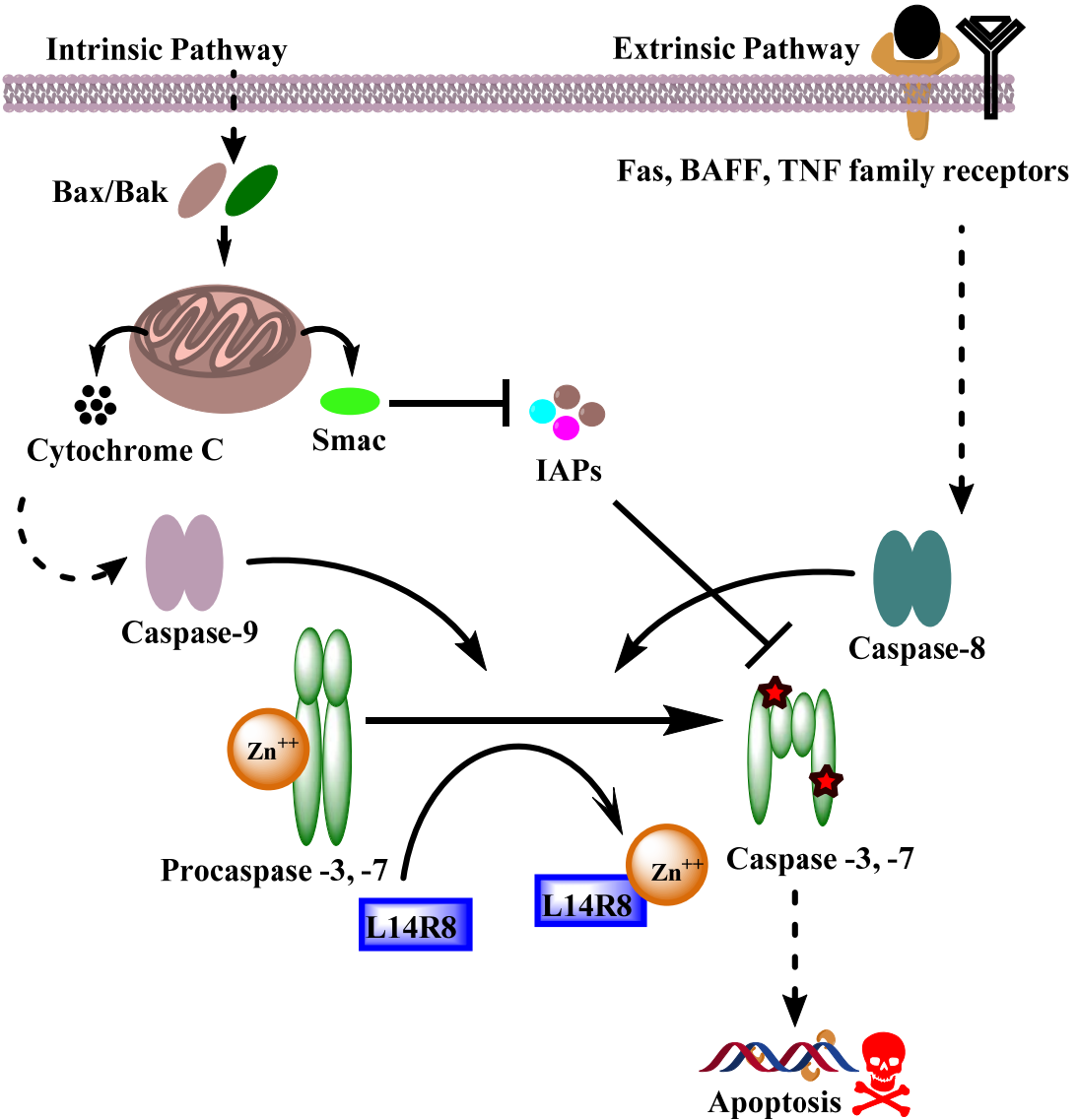
5.2.3: Zinc chelation and selective caspase activation

Zinc ion has also been shown to inhibit procaspase-9 and zinc ion binding sites have been identified on procaspase-9 [174]. Hence, it is possible to predict activation of procaspase 9 by L14R8. However, our observations suggest that L14R8 does not directly activate procaspase-9. This is based on following data. First, procaspase-9 exists as monomer and activation requires dimerization of monomers of caspase-9 while executioner caspases are in dimer states and directly get activated by zinc ion removal. Second, when exogenous zinc was added with ABT-199 to CLL lymphocytes (Figure 40A), ABT-199 mediated cell death was not blocked. ABT-199 induces hallmark features of cytochrome C release, activation of caspase-9 through inhibition of Bcl-2 anti-apoptotic protein [128], and cleavage of procaspase 9 to active caspase 9. If activation of procaspase-9 was zinc ion dependent, addition of exogenous ion along with ABT-199, should interfere ABT199-induced apoptosis. In our experiment, the activity of Bcl-2 antagonist (ABT-199) that acts through the mitochondrial pathway and caspase-9 activation was not impeded by the addition of exogenous Zn ion, suggesting that the activation of caspase-9 is through inhibition of Bcl-2 anti-apoptotic proteins, but not through the removal of labile Zn ion. This indirectly indicates that activation of caspase-9 is not dependent on removal of labile zinc ion on caspase-9. In addition, pan-caspase inhibitors only partially recovered L14R8-mediated apoptosis (Figure 41). Pan-caspase inhibitors bind to catalytic binding sites of executioner caspases and two zinc ion binding sites present on executioner procaspases. It is not clearly known so far how zinc ion removal leads to structural changes, affects the catalytic sites and cleavage of procaspases [174].

Previously, biochemical assays showed that PAC-1 and L14R8 cleaved procaspase-3 was dependent on zinc ion removal [140]. Here, we also demonstrated zinc ion dependent L14R8-induced apoptosis in CLL cells (Figure 40A-B). Moreover, we also demonstrated that L14R8-

mediated apoptosis was through direct activation of caspase-3 and caspase-7 (Figure 37). Hence,

we proposed Figure 49 as a model of mechanisms of L14R8 induced caspase activation.

Figure 49: Proposed mechanism of L14R8 induced procaspase-3 and -7 activation
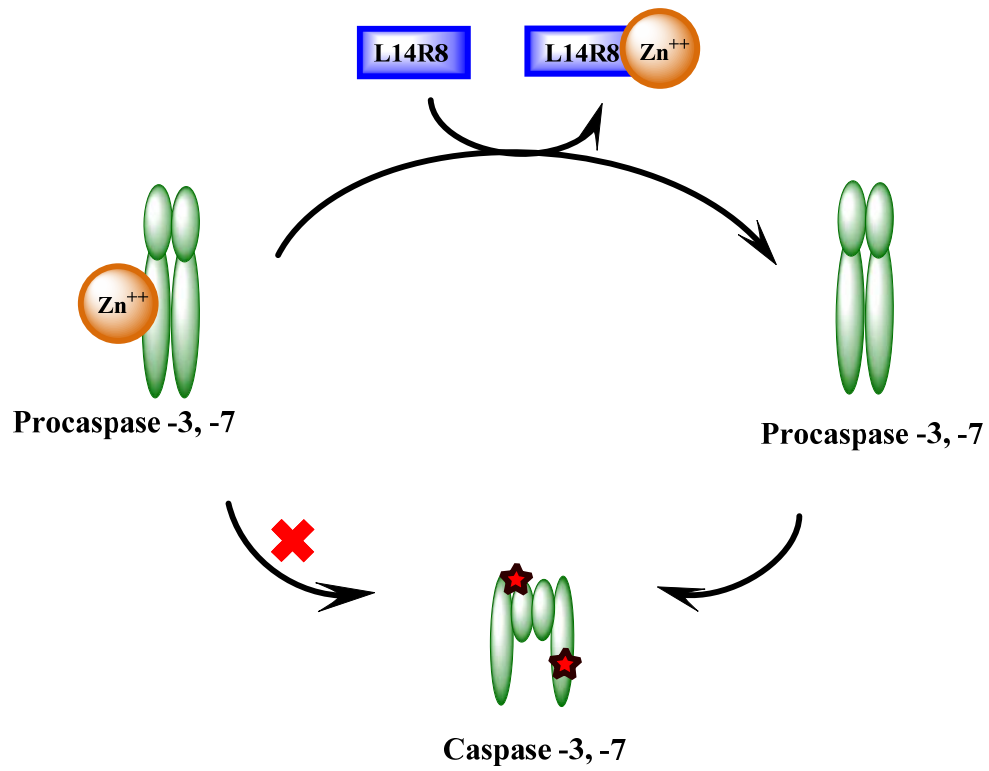
5.2.4: Role of IAPs and Smac mimetics in CLL cell death

First part of study showed comprehensive analysis of changes in expressions of anti-apoptotic Bcl-2 family proteins under stromal cells [148]. Anti-apoptotic Bcl-2 family proteins interact with BH3 helix of pro-apoptotic proteins [175] and interrupt pro-apoptotic proteins-mediated release of cytochrome C and smac proteins from mitochondria [176],[177]. Diablo/Smac protein inhibits IAPs and removes IAPs-mediated caspase-3 inhibition [151]. CLL cells expressed high levels of IAPs and low expression of smac protein compared to healthy donor B-cells and having default caspase-dependent apoptosis [35],[38]. CLL lymphocytes showed higher expression of Ciap1 and Survivin compared to normal lymphocytes and correlated with worse outcome of treatment [38]. Our study supported this notion. First, higher expression of Survivin proteins correlated with resistance to L14R8-induced apoptosis in CLL cells (Figure 44). Second, Survivin and Ciap1 protein expressions were further increased after L14R8 treatment in CLL cells (Figure 43). It might be possible that increased Survivin and Ciap1 expressions after L14R8 treatment would inhibit caspase activity and further apoptosis. With these collective observations, we postulated that smac mimetics will synergize with L14R8. Our data, although limited, were consistent with our postulate.

5.2.5: L14R8 cytotoxicity on normal cells

L14R8 induced significant apoptosis in CLL at 10 µM IC_{50} level (Figure 28). However, 5 µM L14R8 induced cell death in wild type Jurkat cells and MEF cells (Figure 36-38). Compared to malignant CLL cells, L14R8 treatment produced much lower cytotoxicity to pure B cells and PBMCs from healthy donors (Table 7). Collectively, these data suggest a therapeutic index for L14R8 and point toward in vivo testing of this agent in animal system such as CLL mouse models.

5.3: Limitations and future Directions

The investigations in this dissertation provide important sets of information to predict role of apoptosis pathway in CLL cells and changes due to microenvironment. The study also predicts interactions of pro-apoptotic and anti-apoptotic Bcl-2 family proteins leading to CLL cell survival. However, there are certain limitations of the research which provide future direction of the investigations to find better therapeutics and to understand CLL tumor biology.

Previously, many theories demonstrated to predict the CLL cell fate for apoptosis based on the contexts of pro- and anti-apoptotic proteins. Recently new models [162] showed that the simple balance of these proteins is not the only factor which decides the overall fate of the cell and the phenomenon is much more complex than previously predicated. However, the picture of overall prediction of pro- and anti- apoptotic proteins provides the basis to further understand molecular interactions among these proteins. In the present study, we quantitated expression of transcripts and proteins of both pro- and anti- apoptotic proteins at primary level. We also provided a minor attempt below to show interactions of Bcl-2 family pro- and anti-apoptotic proteins based on the analysis of 18 Bcl-2 family members under stromal microenvironment (Figure 47). However, we have not evaluated mechanisms of these changes leading to survival changes in CLL cells. Transcripts expressions of these members reflect the expression pattern of proteins to some extent. However, factors such as post translational modification and turnover of proteins load partly implies mechanistic inputs in CLL survival (reviewed in [178]). Evaluation of these processes needs to be carried out to illustrate comprehensive mechanisms of CLL survival under microenvironment.

We used NKTert cell line, a representative cell line for bone marrow stromal microenvironment. The study revealed that both survival and global RNA expressions were

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increased with stromal coculturing in CLL cells. Another approach to evaluate results would be to carry out analysis in CLL cells under other bone marrow stromal cell types such as ST-2, KUM4, KUSA H1. We choose NKTert cells based on the previous report by Kurtova et al. that NKTert cells provided greater survival advantage among other stromal cells [42]. Apoptosis pathway changes under other stromal marrow cells and accessory cells would confirm the results as generic singling response in survival of CLL under diverse microenvironment.

The CLL-stromal co-incubation model used in the study had limitations. First, we had used CLL, stromal cells ratio of 1:100 for co-culturing. In patient, the ratio is different. Second, stromal cells partially represent bone marrow microenvironment and not lymph nodes and spleen. Third, there are other cells than stromal cells in bone marrow. Fourth, NKTert cell line, even though we used human fibroblast, is immortalized cell line and may not fully represent bone marrow stroma.

Our study demonstrated increased expression of anti-apoptotic proteins in CLL cells under stromal co-culturing, which along with previous reports support the notion that resistance to current treatments occurs due to residual disease in bone marrow. Moreover, other than stromal microenvironment, BCR signaling pathway is another major factor contributing to malignant B cell survival; this is described in the introduction (chapter 1 section 1.1.6 and 1.2.2). The current investigation did not evaluate the apoptosis resistance and survival signals imparted due to BCR signaling.

Here, in the study we had used pre-printed 93 apoptosis genes on the real time RT-PCR array card. However, there are additional genes belonging to different families of apoptosis pathway, which were not included and need to be studied. Few of them are NF-kB family genes, death domain family genes and ligands of the TNFRSF family, which should also be studied.

We focused on impact of stroma on apoptosis pathway in CLL cells; however, there are effects of CLL cells on stroma. As described in introduction (chapter 1 section 1.1.4), CLL and stromal cell interactions involved cysteine transport for glutathione synthesis in drug resistance and prolong survival. Proteins induced in stroma due to interaction with CLL cells, are yet another area of study.

The second part of the dissertation focused on activation of apoptosis in CLL cells by pharmacological activation (l14R8) that bypasses Bcl-2-mediated anti-apoptotic resistance. Our results indicate that in the presence of stromal microenvironment there was reduction in drug-induced apoptosis. It would be important to evaluate role of increased IAP family proteins in inducing resistance L14R8-mediated CLL cell death.

Eventhough detailed cellular and biochemical mechanistic studies were performed. Further evaluations at molecular level of the zinc cleavage site on the executioner caspases are needed. Labile zinc present on procaspases has been important topic of research nearly past two decades [179]. Presence of binding sites on procaspases would provide structural changes of procaspases to better understand molecular biology involved in mechanisms of procaspases activating compounds, which would help to optimize and design generation compounds with better structure-activity relationship.

At another level, physiological level of zinc in human plasma would be important for pharmacological consideration of L14R8 in patients during therapy. Many patients use zinc as supplementary therapy and hence its level may alter L14R8 action. Hence, L14R8 therapy should be staggered with zinc supplement. This avenue needs to be studied using mice models.

Cytotoxic effects of L14R8 and smac mimetics was synergistic. This clearly indicated critical role of IAPs for output of L14R8 treatment. In patients, CLL cells are in constant communication

with stromal cells and hence, stroma induced higher expression of IAPs would affect L14R8

treatment output. Hence, the study of synergy of L14R8 and smac mimetics should be carried out in

the presence of stroma in CLL cells, which would give further directions of development of these

kinds of agents in CLL therapeutics.

While our investigations focused only on smac mimetics in combination with L14R8, other

combinatorial approaches are worthy to test. For example, cytotoxic agents, such as fludarabine

and cyclophosphamide are CLL standard of care for CLL, will induce genotoxic stress [180] and

initiation of intrinsic cell death and activation of procaspases. L14R8 may be able to further

enhance/activate this cell death process. Similarly, novel agents such as ABT-263 or ABT-199 that

target Bcl-2 anti-apoptotic family of proteins [128], need to be evaluated in conjunction with L14R8.

Finally, agents that impede BCR pathway and induce lymphocytosis in peripheral blood of patients

with CLL [181] need to be tested with L14R8. L14R8 may induce cell death of these peripheral blood

malignant CLL cells.

Clinical implication of procaspase compounds in CLL patients should be tested for this novel

compound. First, as mentioned in 5.2.6 section on cytotoxicity on normal cells, mouse models of

CLL would provide important information regarding drug pharmacokinetics, toxicity, efficacy, and

feasibility in human clinical trials. This could be done using TCL-1 transgenic mouse models. If the

toxicity of the current drug is a hindrance, safe and more selective procaspase activating

compounds need to be designed and developed to validate executioner procaspase in leukemia B

cells as an important therapeutic target.

5.4: Conclusion

In summary, first part of our study investigated apoptosis pathway in CLL patient lymphocytes and impact of stromal microenvironment. Our investigations detailed comprehensive analysis of expression profiles of Bcl-2 family proteins in CLL under stromal microenvironment. Our analysis of apoptosis pathway was carried out for short and long duration co-culturing, which showed different pattern of modulation of pro- and anti- apoptotic members and suggested time-dependent molecular interactions in CLL-stromal system. The study also provided information to continue exploring apoptosis resistance and survival signaling mechanisms in CLL cells. In conclusion, our study provided valuable information of apoptosis signaling pathways to understand CLL-bone marrow microenvironment interaction. A corollary to circumvent anti-apoptotic proteins-modulated drug resistance is to treat CLL cells with agents that activate terminal proteins in cell death pathways such as executioner caspases. Second part of study investigated a novel activation approach for defective apoptosis in CLL cells. Our work provided evidence for cytotoxic actions of a novel procaspase activator, L14R8 and elucidates utility of this novel approach for CLL lymphocytes. Therapeutic index and plasma levels of the drug suggest clinical feasibility of this approach. Mechanistic studies illustrated role of zinc ion while mechanism based combination approaches suggested IAP family.

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VITA

Viralkumar Patel was born in Nadiad, Gujarat, India on December 25, 1985 to parents of Manaharbhai Patel and Prafullaben Patel as a second child. He grew up in Arajanvav, a small village in Bayad Taluka at Sabarkantha district in the state of Gujarat in India. He attended N.H. Shah High School in Bayad Town, from 1990-2000. He finished his remaining schooling at A-One High School, Ahmedabad, Gujarat, India during 2001-2003. Viral has achieved his Bachelors of Pharmacy at Sardar Patel University, VV Nagar, India. Viral came to Grand Valley State University, Allendale, MI as a Master's student in August 2007 and finished Masters in Cellular and Molecular Biology in July 2009. In August 2009, Viral enrolled at the University of Texas, Graduate School of Biomedical Sciences at Houston, and later joined the laboratory of Dr. Varsha Gandhi in the Department of Experimental Therapeutics at MD Anderson Cancer Center, and became a member of the Experimental Therapeutics Academic Program (ETAP). Viral conducted his Ph.D. work under Dr. Gandhi's supervision, and he will complete all requirements of Doctor of Philosophy degree in December, 2013. During Viral's Ph.D. training, he received multiple travel awards from both UT-GSBS and ETAP program to attend the annual conferences of American Association of Cancer Research (AACR) where he presented his research projects. In addition, he received a Young Investigator Awards from American Association of Indian Scientists in Cancer Research in 2011.

Publications:

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Permanent address:

At-Arajanvav, Post-Madhavkampa,

Taluka-Bayad, District-Sabarkantha,

Gujarat-383325, India