

DEVELOPMENT OF AN IMMUNOCYTOCHEMISTRY PROTOCOL FOR THE
STUDY OF TAL1 MEDIATED APOPTOTIC RESISTANCE IN JURKAT CELLS

A RESEARCH PROJECT

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INTRODUCTION

Hematopoietic stem cells (HSCs) differentiate into blood cells of the body during hematopoiesis [1, 2]. HSCs are characterized by the capacity to self-renew, maintain, and differentiate into common lymphoid or myeloid progenitors in response to stimulation by growth factors and cytokines [3, 4]. Lymphoid and myeloid progenitors differentiate into the blood, bone marrow, spleen, or thymus cells of the body, regulated by transcription factor proteins [2]. Transcription factors are essential during hematopoiesis [5], and regulate activation and repression of genes at specific times, guiding the proliferation, differentiation, or quiescence of HSCs [6]. A master regulator of hematopoiesis, TAL1 is required for development and differentiation of HSCs during embryogenesis, after which, it is inactivated [7]. The importance of TAL1 is demonstrated in that embryos lacking functional TAL1 are unable to develop hematopoietic cells, resulting in anemic death [8-10].

TAL1 is a basic-helix-loop-helix transcription factor that regulates development of all hematopoietic cells by activating, and repressing gene transcription [6]. The exact mechanisms by which TAL1 influences gene expression remain poorly understood, but regulation is linked to DNA binding, recruitment of coactivators, or corepressors to promoters, and binding other transcription factors [7]. TAL1 forms heterodimers with ubiquitously-expressed E-protein transcription factors (e.g. GATA1, LMO1/2, RUNX3) [6]. TAL1 heterodimers translocate to the nucleus to bind E-box, GATA, and CACC consensus sequences to regulate gene transcription [11]. Activation, or

repression of transcription, depends on what heterodimer is formed, as well as the association of coactivators (e.g. p300, PCAF), or corepressors (e.g. LSD1, HDAC1/2), with the TAL1 complex [6, 12]. When the TAL1 complex associates with corepressors, repression of gene transcription occurs by recruitment of histone deacetylases (e.g. HDAC1/2) [12]. Deacetylation of histones causes chromatin condensation, resulting in loss of transcriptional activity of those genes in proximity to the event. The TAL1 complex activates gene transcription when associated with coactivators (e.g. p300, PCAF), which recruit histone acetyltransferases to acetylate the lysine tail of histones, causing histone dissociation from DNA. Dissociation of histones from DNA causes chromatin to decondense, allowing proteins needed for transcription to interact with gene promoters. Conversely, TAL1 mediates methylation of histone proteins to prevent acetylation, maintaining certain genes in an inactive state. TAL1 also regulates cellular processes by directly binding with, and inhibiting, other proteins [11, 13]. Regulated functioning of TAL1 is essential [5], as it influences multiple cellular processes during hematopoiesis, such as differentiation, proliferation, and survival, through activation, or repressive mechanisms [10, 13]. Ectopic expression of TAL1 can contribute to malignant conditions by deregulating transcriptional networks, such as with T-cell acute lymphoblastic leukemia (T-ALL) [6, 9].

T-ALL is an aggressive cancer resulting from oncogenic mutations in genes regulating cell cycle, differentiation, and survival of progenitor T-cells [9]. T-ALL constitutes roughly 15% of pediatric and 25% of adult ALL, and is the most

frequently diagnosed malignancy found in children [14]. Male children with a median age of 12 years comprise the largest group diagnosed with T-ALL. T-ALL is clinically characterized by the presence of undifferentiated T-cell lymphoblasts within the bone marrow, elevated white blood cell counts, central nervous system invasion [9], and suppression of normal hematopoiesis [14]. The development of clonal populations of maturation-arrested leukemic cells within the bone marrow is followed by metastasis into non-hematopoietic tissue. Few treatment options are available for patients diagnosed with primary resistant or relapsed T-ALL, which maintain poor prognosis due to treatment resistance [9]. Successful treatment of T-ALL requires intensive chemotherapy [15], and can result in life-threatening acute, or chronic toxicity complications [16]. The exact causes leading to T-ALL development are not characterized fully, but radiation and carcinogen exposure are correlated with increased risk of development [14]. Multiple mutations contribute to T-ALL progression, such as those involved with cell signaling, cell cycle control, tumor suppression, signal transduction [8], chromatin remodeling, and transcription factors, such as TAL1 [9]. Poor prognosis, limited treatment options, and life-threatening treatment complications substantiate the need for more effective treatments [9]. Understanding the molecular relationship that exists between T-ALL and TAL1 can help identify potential novel targets for chemotherapy.

The ectopic expression of *TAL1* is found to occur in roughly 60% of patients with T-ALL [9], and is the most frequent abnormality associated with this type of leukemia [6, 12]. The *TAL1* gene is inactivated following hematopoiesis,

but gain-of-function mutations in *TAL1* lead to its reactivation, and overexpression in T-cell progenitors [6, 9, 14]. The mechanisms causing reactivation of *TAL1* remain poorly understood, but can be due to chromosomal mutations [6], oncoprotein mediated activation of *TAL1* (e.g. c-MYC) [17], or aberrant chromatin looping [18]. A 90kb DNA interstitial chromosome deletion at 1p32 occurs in roughly 30% of T-ALL cases [9]. The 1p32 deletion reactivates, and causes overexpression of *TAL1* by placing it under the influence of the highly expressed *STIL* promoter [19, 20]. Reactivation due to chromosomal translocations, such as t(1;14)(p32;q11) or t(1;7)(p32;q34), place *TAL1* under the influence of active *TCRA/D* enhancers [9, 20]. *TAL1* directly targets genes contributing to T-ALL progression, such as *GATA1/3*, *LMO1/2*, *MYB*, *MYC*, *CCNE*, *NKX3.1*, *NOTCH1*, *RUNX1/3*, and *TRIB2* [9, 13, 21, 22]. Additionally, *TAL1* progresses T-ALL through inhibiting the *CDKN2A* gene, which encodes a critical cell cycle control protein, p16INK4A, as well as preventing T-cell differentiation by inhibiting the *pTα* gene [23]. The ectopic expression of *TAL1* is linked to chemotherapeutic resistance [24-26], as it influences proteins involved in apoptosis and survival [13, 27-29].

Apoptosis is the programmed death of a cell by activation of either intrinsic (mitochondrial-based), or extrinsic (death receptor) apoptotic pathways in response to specific stimuli [30]. Internal cellular stressors, such as oxidative stress, or irreparable DNA damage lead to activation of the intrinsic apoptotic pathway. Extracellular signals, such as FasL or TNF ligands, bind with death receptors to activate the extrinsic apoptotic pathway. Characteristics of apoptosis

include chromatin condensation, DNA fragmentation, cytochrome c release, cellular shrinkage, mitochondrial degradation, as well as nuclear and membrane blebbing. Apoptosis is critical to the prevention of tumorigenesis, and propagation of cells that have undergone detrimental genetic mutations. Both intrinsic and extrinsic pathways lead to the same general biochemical changes, caspase activation, degradation of DNA and proteins, as well as, changes in membranes to allow for recognition and phagocytosis by macrophages. Unlike necrosis, apoptosis does not result in an inflammatory response. Proper functioning of apoptotic proteins (e.g. BID, caspase-9, PARP) is essential for prevention of leukemogenesis, and efficacy of treatments. Deregulation can result in cells resisting apoptosis.

The exact mechanisms by which TAL1 influences survival have yet to be identified and characterized [6, 10]. The inability of HSCs to survive without functional TAL1 demonstrates a role in apoptosis [31]. TAL1 potentially mediates cell survival by influencing the function of proteins involved in apoptosis, complexing with proteins known to have pro-survival functions (e.g. GATA1) [32], or by suppressing genes encoding pro-apoptotic proteins [11, 33, 34]. The pathways in which TAL1 may suppress apoptosis are not yet characterized. TAL1 binds with, and increases, activity of the *c-KIT* promoter [7], influencing signaling and survival through upstream, and downstream, actions within the c-KIT pathway [7, 33]. Nineteen genes encoding apoptotic proteins (e.g. *API5*, *BIRC2*, *CUL5*, *TGFB1*, and *SMNDC1*) are co-modulated by TAL1, and the c-KIT kinase [33]. TAL1 may influence survival through the VEGF pathway, where it

suppresses apoptosis during hematopoiesis [35]. TAL1 involvement in suppressing apoptosis is supported by its up-regulation of genes encoding miRNAs, such as miR-223 and miR-330 [28, 29]. MiR-223 suppresses expression of pro-apoptotic proteins, such as E2F1, FOXO1, and FBXW7. MiR-330 suppresses apoptosis inducing proteins E2F1 and CDC42 [29]. TAL1 directly up-regulates the expression of the protein, TRIB2 [11], as well as indirectly, by inhibiting TRIB2 repressors, E2A and HEB [13]. TRIB2 inhibits FOXO tumor suppressor proteins and increases the malignant phenotype of cells [11, 36]. Aberrant TAL1 expression is linked to the deregulation of the apoptotic process in T-ALL, but its influence on apoptotic proteins, Bcl-X_L, caspase-9, and PARP have yet to be determined. The use of immunocytochemistry may help elucidate the role played by TAL1 in modulating the expression and function of these apoptotic proteins.

Immunocytochemistry is a technique utilized in fluorescence microscopy to study protein interactions, functions, expression, localization, and abundance [37]. Primary antibodies, raised against a specific protein of interest, can cross the cellular plasma membrane following permeabilization, and bind to a specific protein of interest [38]. Secondary antibodies, raised against the species in which the primary antibodies are raised, then are introduced and will bind the primary antibody. Additionally, the secondary antibody is bound with a fluorochrome, such as FITC, which will emit fluorescence when excited by exposure to the appropriate wavelength of light. Fluorescence microscopy utilizes these specific wavelengths of light to bombard the cells, causing excitation of the fluorophore

attached to the secondary antibody, enabling the visualization of the intended protein of interest. This method can be used in the localization of proteins within cells, as well as, to analyze the level of expression of proteins of interest. These analyses can be used to further the understanding of various conditions, such as leukemia. Identification of protein expression and localization within the cell can be used to help identify potential abnormalities of cells, possibly culminating in the identification of potential novel chemotherapeutic targets. The technique of immunocytochemistry also can be used to study cellular processes, such as nuclear translocation of proteins. Cellular nuclei can be visualized through the use of nuclear counterstains, such as DAPI [39]. DAPI will stain the DNA of cells by binding DNA regions rich in A-T sequences, enabling the identification of nuclei via fluorescence. This enables the identification of nuclear localized proteins, or proteins that translocate to, or from the nucleus, such as NUR77 or NF κ B/p65, in response to certain stimuli by analyzing the migration and localization of protein fluorescence in relation to the nucleus.

Immunocytochemistry can be limited by background fluorescence and non-specific antibody binding. However, through the use of blocking agents, this non-specific binding can be mitigated. Immunocytochemistry offers a valid method for detecting protein expression and localization, as well for the study of various cellular processes or responses of cells to treatments. The development of an immunocytochemistry protocol could help further the understanding of how TAL1 influences death-associated proteins, such as the NF κ B family of transcription factors.

The NF κ B protein family encodes transcription factors that possess both anti- and pro-apoptotic functions [40]. The TAL1 transcription factor has been shown to directly bind with the Ebox consensus sequences of NF κ B genes, and thus, modulate transcription of proteins within this family [41]. This modulation can lead to the overexpression of pro-survival NF κ B proteins, such as NF κ B/p65, leading to the suppression of apoptosis and cancerous cells resisting chemotherapy. The expression of death-associated proteins, such as NF κ B or Bcl-X_L, are associated with being influenced by the expression of TAL1. This makes the identification of the relationship that exists between TAL1 and these proteins important in elucidating the mechanisms by which TAL1 promotes the suppression of apoptosis during chemotherapeutic treatment of T-ALL.

The Bcl-2 (B-cell lymphoma-2) protein family consists of pro- and anti-apoptotic proteins that function in regulating the intrinsic apoptotic pathway, but also can be activated by the extrinsic apoptotic pathway [30, 42]. Bcl-2 proteins form homo- or heterodimers via BH3 (Bcl-2 homology domain 3) domains to regulate the apoptotic response [30, 43]. Initiation of apoptosis is determined by the ratio of pro-survival Bcl-2 proteins (e.g. Bcl-2, Bcl-X_L) to pro-apoptotic (e.g. BID, Bad). Stressors, such as irreparable DNA damage, may lead to increased transcription and activation of pro-apoptotic members, resulting in oligomeric pore formation in the mitochondrial membrane, and subsequent cytochrome c release [30]. Central to this process is the pro-apoptotic protein BID, which is activated upon cleavage by the protease, caspase-8, into the truncated form, tBID [43]. Once cleaved, tBID translocates to the outer mitochondrial membrane

where it binds with, and inhibits Bcl-2, causing the release of Bax and Bak proteins [44]. Bax and Bak form oligomeric pores in the mitochondria, releasing cytochrome c. Oncogenic mutations in cancerous cells may lead to the overexpression of pro-survival Bcl-2 proteins, resulting in cancerous cells resisting apoptotic induction and decreasing the efficacy of chemotherapy [45, 46]. Release of cytochrome c into the cytosol leads to activation of caspases, such as caspase-9, needed to carry out the further downstream events of the apoptotic process [30, 44, 47].

The initiator proteases, such as caspase-8, -9, are important to the induction of apoptosis and efficacy of chemotherapeutic treatments and their proper functioning is essential [30]. Caspases mediate the controlled degradation of the cell via the caspase cascade, which can be activated by either extrinsic (receptor based) or intrinsic (mitochondrial based) apoptotic pathways. For example, caspase-9 is activated in the intrinsic apoptotic pathway after the release of cytochrome c from the mitochondria [30]. Cytochrome c binds with and activates the monomeric form of the adaptor protein, Apaf-1 [48]. Apaf-1/cytochrome c undergoes a conformational change using energy derived from ATP hydrolysis, enabling it to oligomerize with other activated Apaf-1/cytochrome c complexes to form the heptameric apoptosome. Apaf-1 recruits and binds with the pro-domain of pro-caspase-9 via an N-terminal caspase recruitment domain, forming the apoptosome complex [47, 48]. Apoptosome complexes position pro-caspase-9s in close proximity to each other, enabling autoproteolytic cleavage of pro-caspase-9s into the active cleaved form. Cleaved caspase-9s will

heterodimerize, and proceed to cleave the effector protease, pro-caspase-3 into its active form, cleaved caspase-3 [30]. Activation of caspase-3 results in the cleavage of numerous substrates, such as cytoskeletal proteins, kinases, and proteins involved in DNA repair, such as the protein PARP [30, 49].

The process of detecting and repairing DNA single-strand breaks and nucleotide damage is dependent upon the PARP family of proteins [49]. PARP enzymes function in DNA damage detection, cell cycle arrest, single-strand break repair, and base excision repair pathways [49]. Upon detection of DNA damage, PARP binds DNA using N-terminal zinc finger structures similar to that of DNA ligase. DNA binding activates the catalytic C-terminal domain of PARP[50], which then synthesizes an ADP-ribose polymer from an NAD^+ substrate [49]. Poly(ADP-ribosyl)ation serves dual functions, providing both a cellular signal of DNA damage, which recruits key DNA repair proteins, DNA polymerase- β , DNA ligase, and XRCC1 to the damaged site, as well as inducing the relaxation of chromatin structure by causing histone dissociation from DNA. PARP also functions in mediating the induction of apoptosis should DNA damage be irreparable through the release of the mitochondrial flavoprotein, apoptosis-inducing factor (AIF) [51]. AIF indirectly activates caspase-9 by causing release of cytochrome c from the mitochondria. AIF also translocates to the nucleus, where it facilitates chromatin condensation, and DNA fragmentation in chromatinolysis (caspase-independent apoptosis) [52]. The multiple roles of PARP in DNA repair, as well as its ability to induce chromatinolysis, provide reason for TAL1 to spare PARP from degradation.

The prognosis of patients afflicted with treatment resistant T-ALL remains poor [9]. While the affects of TAL1 in the hematopoietic process are somewhat characterized, its abilities to facilitate apoptotic resistance by influencing death-associated proteins, such as Bcl-X_L, caspases, NF κ B, and PARP, currently are not understood. In this study, we propose to develop a protocol for immunocytochemistry, in order to examine the response of Jurkat cells to apoptotic stimulation using etoposide. The Jurkat T-cell line was derived from a patient with treatment resistant, relapsed T-ALL [53], and is used as a model to study T-cell signaling and drug treatment response. These reasons provided the basis for the selection of the Jurkat cell line for use in the present research study [54, 55]. This will enable future studies to better identify and define the role of certain proteins in T-ALL. Elucidating the role of TAL1 in mediating apoptotic resistance will enable the identification of possible chemotherapeutic targets, and potentially help lead to the development of more effective drugs for use in the treatment of T-ALL.

METHODS

Cell Culture

Jurkat cells will be cultured in T-25 flasks (Sarstedt) using RPMI 1640 + L-Glutamine (Life-Technologies)/10% BGS (Thermo-Fisher) media. Cells will be cultured at 37° C in a Forma Scientific 5% CO₂ incubator. Cells will be sub-cultured and medium replaced three times weekly, or as needed for experimentation. Cell counts will be performed using the trypan blue exclusion

method, as previously described in Altman et al. [56]. Cell counts will be performed by resuspending cultures and transferring cell suspension to a microcentrifuge tube containing trypan blue (Sigma). The cell suspension then will be resuspended and added to a dual-chamber hemacytometer (Hausser Scientific) for counting using a Nikon inverted light microscope (model TMS). Cell counts will be determined by counting total unstained cells present in four corners, dividing to obtain the average, and multiplication of the average by dilution and volume conversion factors.

Drug Treatment

Cells will be drug-treated (24 hrs) using etoposide (Enzo) at concentrations of 1 μM and 5 μM ($n = 3$ per group) as previously described (SEM thesis, 2013). The 1 μM treatment mirrors the concentration used in chemotherapeutic regimens, while the 5 μM is known to induce wholesale apoptosis in cells (unpublished results, SEM). Additionally, a group of untreated cells ($n = 3$) will serve as a control. Cell counts will be performed as previously described and then 5×10^5 cells/mL (per group) will be spun for 5 min using a Fisher-Scientific tabletop centrifuge (model Centrifric) at 1000 rpm at room temperature. Media will be aspirated, and cells resuspended in RPMI 1640/10% BGS, RPMI 1640/10% BGS/1 μM etoposide, or RPMI 1640/10% BGS/5 μM etoposide according to group. Following resuspension, cells will be incubated (24 hrs) at 37° C and 5% CO₂ in a Forma Scientific incubator. All samples will then be examined using immunocytochemistry.

Immunocytochemistry

Immunocytochemistry will be performed on control untreated, 1 μ M and 5 μ M etoposide-treated Jurkat cells. Etoposide treatment will be performed as previously described. Cells will be fixed for immunocytochemistry using a protocol modified from Lyss et al. [57]. A cell suspension of 5×10^5 cells/mL (per group) will be spun for 5 min at 1000 rpm at room temperature, the culture media aspirated, and cells resuspended in an equivalent volume of 1X PBS (Life-Technologies), followed by centrifugation for 5 min at 1000 rpm at room temperature. Next, cells will be air-dried for 1 hr on slides. Cells will then be fixed at room temperature for 15 min by adding a drop of 4% paraformaldehyde (Amresco) and applying a plastic coverslip. After fixing, the slides will be rinsed for 2 min in a Coplin jar containing 1X PBS and cells permeabilized by incubating for 10 min in 1X PBS/0.5% Triton X-100 (Sigma). Excess solution will be removed and samples incubated for 20 min in 5% goat serum (Thermo-Fisher)/0.5% BSA (Amresco)/1X PBS solution at room temperature. Next, samples will be incubated for 1 hr using either a mouse, or rabbit primary antibody diluted according to the manufacturer recommendations in 1X PBS. Slides will then be washed for 5 min in 1X PBS/0.01% Triton X-100. Excess primary antibody solution will be removed, and samples incubated for 1 hr in either anti-mouse Alexa Fluor 488 secondary antibody (Cell Signaling) or anti-rabbit Alexa Fluor 488 secondary antibody (Cell Signaling) diluted 1:1000 in 1X PBS. Next, samples will be washed for 5 min in 1X PBS. Excess secondary antibody solution will be removed and a few drops of ProLong Gold antifade with DAPI (Life-Technologies) will be added. Coverslips will be mounted and slides

left to cure for 24 hrs overnight. All sample groups will utilize additional no antibody, primary antibody only, and secondary antibody only controls. Slides will be stored at 4° C until analysis is performed. Cells will be viewed and images collected using a Zeiss fluorescence microscope (Axioskop 2) fitted with a Zeiss confocal head (LSM 5 PASCAL). Images will be acquired at 100X magnification using LSM PASCAL software.

RESULTS

Detection of β -actin in both untreated and treated samples was identified by green fluorescence localized in the cytoplasm of cells outside of the nucleus (Figure 1). Nuclei were successfully detected using a nuclear DAPI counterstain, which stained the nuclei blue in color (Figure 1). Minimal apoptosis occurred in the control untreated and 1 μ M etoposide-treated Jurkat groups. However, the 5 μ M etoposide-treated sample displayed an increased level of apoptosis when compared to control untreated and 1 μ M etoposide-treated groups.

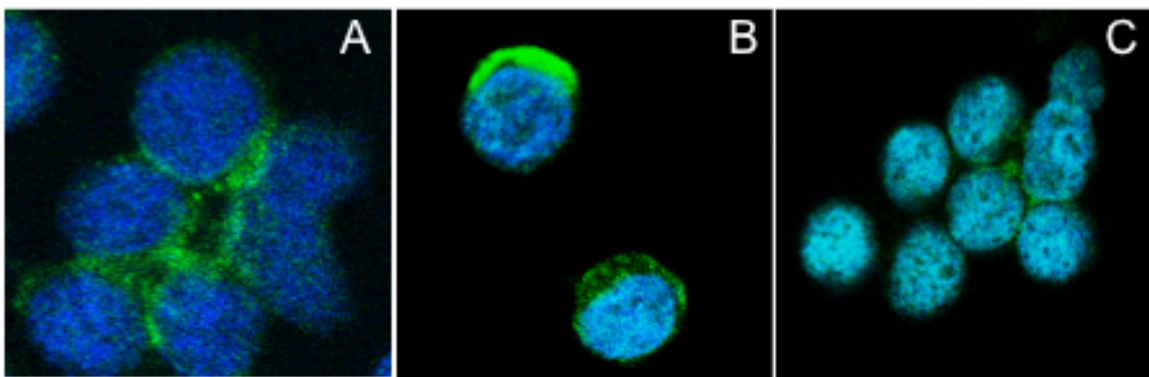


Figure 1. (A) Untreated Jurkat cells displaying β -actin (green) expression in the cytoplasm with minimal localization within the nucleus (blue). (B) When treated with 1 μ M etoposide, β -actin (green) was detected in the cellular cytoplasm. (C) Upon

treatment of cells using 5 μ M etoposide, β -actin remained localized in the cytoplasm of cells. Cells displayed the round morphology characteristic of Jurkat T-cell lymphocytes. All β -actin proteins were detected using a mouse anti- β -actin primary antibody and anti-mouse Alexa Fluor 488 secondary antibody at 488 nm, with nuclei detected using a DAPI counterstain at 405 nm. All images were collected at 100X magnification using a Zeiss fluorescence microscope fitted with an LSM 5 PASCAL confocal head.

In both untreated and etoposide-treated cells, Bcl-X_L expression was identified by green fluorescence and was located juxtaposed in the cytoplasm next to nuclei, which were stained blue by the DAPI counterstain. Minimal apoptosis was identified in both untreated (Figure 2, A) and 1 μ M etoposide-treated (Figure 2, B) samples, but increased levels of Bcl-X_L fluorescence was identified in the 1 μ M etoposide-treated sample. The 5 μ M etoposide-treated cells displayed decreased levels of Bcl-X_L fluorescence, with increased levels of apoptosis, as denoted by nuclear blebbing and fragmentation (Figure 2, C).

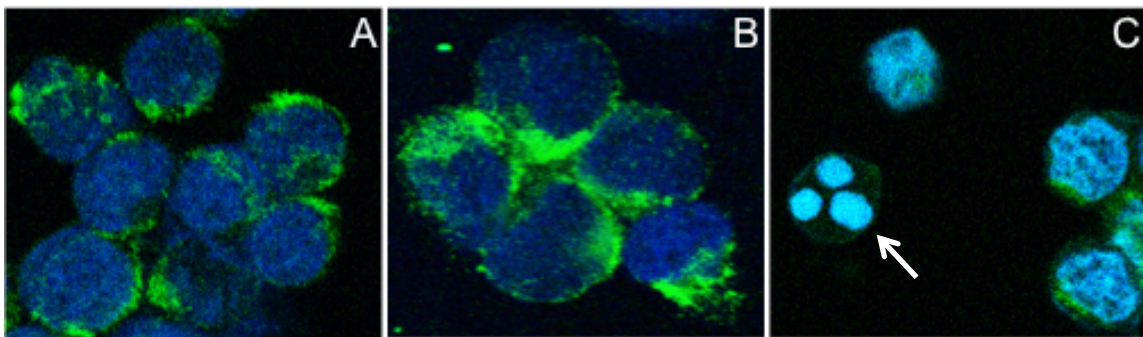


Figure 2. (A) Untreated Jurkat cells displaying Bcl-X_L (green) expression in the cellular cytoplasm with minimal localization within the nucleus (blue). (B) When treated with 1 μ M etoposide, Bcl-X_L (green) was detected juxtaposed to the nucleus (blue), as well as in the cytoplasm, with no nuclear blebbing or fragmentation detected. (C) Upon

treatment of cells using 5 μ M etoposide, Bcl-X_L (green) expression was detected in the cytoplasm of cells, with some nuclei (blue) undergoing nuclear blebbing and fragmentation (white arrow). Bcl-X_L proteins were detected using a rabbit anti-Bcl-X_L primary antibody and anti-rabbit Alexa Fluor 488 secondary antibody at 488 nm, with nuclei detected using DAPI counterstain at 405 nm. All images were collected at 100X magnification using a Zeiss fluorescence microscope fitted with an LSM 5 PASCAL confocal head.

In both untreated and etoposide-treated cells, NF κ B/p65 expression was identified by green fluorescence and was located juxtaposed in the cytoplasm next to the nuclei, which were stained blue by the DAPI. Minimal apoptosis was identified in both untreated (Figure 3, A) and 1 μ M etoposide-treated (Figure 3, B) samples. The 1 μ M etoposide treatment did result in cells displaying greater intensity of green NF κ B/p65 fluorescence, which was ringed around the nuclei and some cells displayed the green fluorescence infiltrating the nucleus (Figure 3, B). No increase in NF κ B/p65 fluorescence was identified in the 5 μ M etoposide-treated sample (Figure 3, C). The 5 μ M etoposide-treated cells displayed increased levels of apoptosis, as denoted by nuclear blebbing and fragmentation (Figure 3, C).

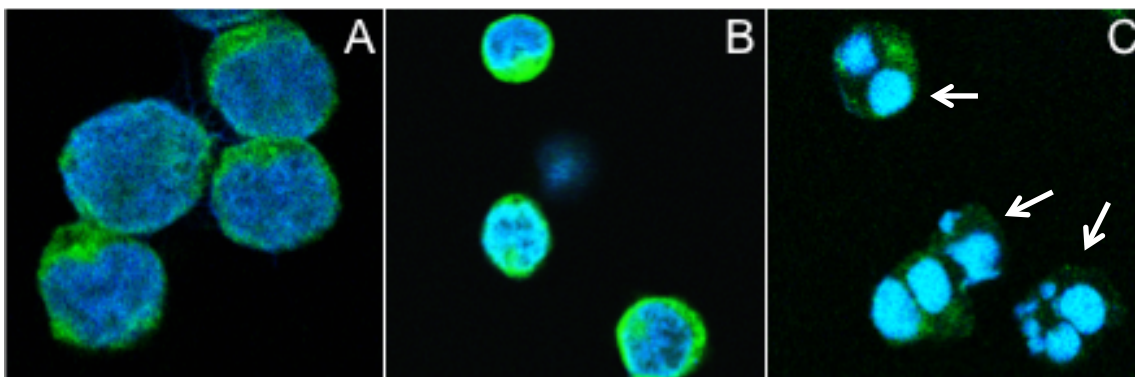


Figure 3. (A) Untreated Jurkat cells exhibiting NF κ B/p65 (green) expression in the cellular cytoplasm with minimal localization within the nucleus (blue). (B) When treated with 1 μ M etoposide, NF κ B/p65 (green) expression was detected juxtaposed or moving into the nuclei (blue) of the cells, with no nuclear blebbing or fragmentation detected. (C) Upon treatment of cells using 5 μ M etoposide, NF κ B/p65 (green) expression was detected in the cytoplasm of cells with nuclei (blue) undergoing nuclear blebbing and fragmentation (white arrows). Cells displayed the round morphology characteristic of Jurkat T-cell lymphocytes. NF κ B/p65 proteins were detected using a rabbit anti-NF κ B/p65 primary antibody and anti-rabbit Alexa Fluor 488 secondary antibody at 488 nm, with nuclei detected using DAPI counterstain at 405 nm. Images were collected at 100X magnification using a Zeiss fluorescence microscope fitted with an LSM 5 PASCAL confocal head.

In both untreated and etoposide-treated cells, PARP expression was identified by green fluorescence and was located throughout the cytoplasm of the cell. Nuclei were successfully stained blue by the DAPI counterstain. Minimal apoptosis was identified in both untreated (Figure 4, A) and 1 μ M etoposide-treated (Figure 4, B) samples. The 1 μ M etoposide treatment did result in cells displaying increased levels of PARP expression, which appeared to be located in

the cytoplasm, as well as having migrated into the nucleus (Figure 4, B). PARP expression was primarily localized in the cytoplasm, not in the nucleus, in the 5 μ M etoposide-treated sample (Figure 4, C). The 5 μ M etoposide-treated cells displayed increased levels of apoptosis, with apparent nuclear blebbing and fragmentation (Figure 3, C).

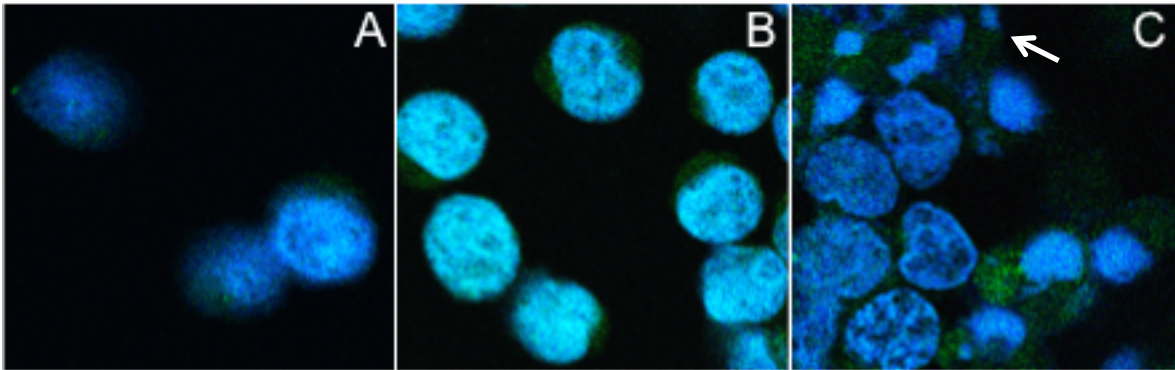


Figure 4. (A) Untreated Jurkat cells exhibiting PARP (green) in the cytoplasm with minimal localization within the nucleus (blue) (B). When treated with 1 μ M etoposide, PARP (green) expression was detected in both the cytoplasm and moving into the nucleus (blue) of the cells, with no nuclear blebbing or fragmentation detected. (C) Upon treatment of cells using 5 μ M etoposide, PARP (green) expression was detected in the cytoplasm of cells with some nuclei (blue) undergoing nuclear blebbing and fragmentation (white arrow). Cells displayed the round morphology characteristic of Jurkat T-cell lymphocytes. PARP proteins were detected using a rabbit anti-PARP primary antibody and anti-rabbit Alexa Fluor 488 secondary antibody at 488 nm, with nuclei detected using a DAPI counterstain at 405 nm. All images were collected at 100X magnification using a Zeiss fluorescence microscope fitted with an LSM 5 PASCAL confocal head.

DISCUSSION

β -actin is a highly conserved, ubiquitously expressed protein that functions in maintaining cell structure, guiding motility, as well as, assisting with cell division, and serves as a control for this study [58]. In agreement with our anticipated results, as well as those of Lyss et al. [57], β -actin was found to be expressed outside of the nucleus in the cytoplasm of cells. Comparison of control untreated, 1 μ M and 5 μ M etoposide-treated samples revealed that stimulation of apoptosis did not alter the localization and expression of actin. All cells displayed a round morphology characteristic of T-cell lymphocytes, which was in agreement with the findings of Alexander and Wetzel [59]. Based on these results obtained, the immunocytochemistry protocol performed was successful in identifying β -actin protein.

Bcl-X_L is a pro-survival member of the Bcl-2 family of proteins and its regulated function is central to the processes involved in the induction, or repression of apoptosis in cells [30]. This study successfully detected Bcl-X_L protein in Jurkat cells. Control untreated cells displayed Bcl-X_L expression throughout the cytoplasm of the cell, primarily ringed around the cell nucleus. Stimulation of apoptosis using 1 μ M etoposide resulted in increased levels of Bcl-X_L fluorescence, both throughout the cytoplasm, as well as juxtaposed to the nucleus. Minimal apoptotic morphology was displayed by cells in the 1 μ M etoposide-treated sample when compared to the control untreated sample. The lack of increased levels of apoptosis in cells overexpressing Bcl-X_L, as well as the increase in Bcl-X_L expression, were in agreement with the findings of Minn et

al. [60]. It was determined that increased Bcl-X_L expression in malignant cells can lead to the decreased efficacy of etoposide to induce apoptosis. Additionally, samples treated using 5 μ M etoposide did not display an increase in Bcl-XL expression and were found to exhibit increased levels of apoptosis, indicated by nuclear blebbing and membrane fragmentation. These results were in agreement with a previous study which found 5 μ M etoposide treatment was sufficient to induce apoptosis in cells (SEM thesis, 2013). The aberrant expression, or functioning of Bcl-X_L, can contribute to cancerous cells developing multi-drug resistance, resulting in decreased efficacy of chemotherapeutic treatments [60]. Identifying if the expression, or functioning of the pro-survival Bcl-X_L protein are influenced by TAL1 can help identify the mechanisms contributing to T-ALL chemotherapy resistance. Thus, potentially leading to the identification of novel targets for T-ALL treatment.

NF κ B proteins belong to a family of transcription factors involved in pro-survival, or pro-apoptotic functions of the cell and can contribute to malignant phenotype and suppression of apoptosis if not properly regulated [40]. The results of the present study found NF κ B/p65 present within the cytoplasm of untreated and 1 μ M etoposide-treated samples. When compared to the untreated control, increased levels of NF κ B/p65 expression were found in the 1 μ M etoposide-treated sample, with fluorescence appearing to migrate into the nucleus. NF κ B/p65 seems to be translocating into the nucleus to facilitate transcription of pro-survival proteins, suppressing the induction of apoptosis in 1 μ M etoposide-treated samples. No increased level of apoptosis was found to

occur in the 1 μ M etoposide treatment sample when compared to the control untreated sample, indicating that cells were effectively suppressing the apoptotic response. This may be due to the potential function and increased expression of NF κ B/p65. The lack of a substantial increase in apoptotic cells was in agreement with previous findings (SEM thesis, 2013), as aberrant expression or functioning of NF κ B/p65 is thought to lead to suppression of apoptosis [61]. The 5 μ M etoposide treatment resulted in no detectable increase in NF κ B/p65 expression, which appeared to be located in the cytoplasm. A substantial increase in cells undergoing apoptosis was observed, as indicated by nuclear blebbing and membrane fragmentation. This was in agreement with previous work, in which 5 μ M etoposide treatment was shown to be sufficient to induce apoptosis in Jurkat cells (SEM thesis, 2013). Identifying the relationship that exists between the TAL1 transcription factor and aberrant expression or function of the NF κ B/p65 protein may potentially lead to identification of novel targets and warrants further investigation.

The PARP family of proteins function in the identification and signaling of DNA damage by poly(ADP-ribosyl)ation, recruitment of repair proteins, and prevention of irreparable DNA damage [49]. Chemotherapeutic agents, such as etoposide, induce DNA damage by inhibiting topoisomerase, effectively inducing cells into apoptosis [62]. Thus, aberrant functioning or expression of DNA repair enzymes, such as PARP, can mitigate the ability of chemotherapeutic agents to induce apoptosis in cancerous cells. The present study examined the expression and localization of PARP in cells stimulated to undergo apoptosis using

etoposide. Untreated control samples displayed minimal PARP expression, which was localized in the cytoplasm of cells. This was in agreement with our anticipated results, as DNA damage was not being induced and PARP repair of DNA damage was not required. However, in cells stimulated to undergo apoptosis using 1 μ M etoposide, increased expression of PARP was found to occur. There appears to be a migration of fluorescence signal (indicative of PARP) into the nucleus, presumably to repair DNA damage induced by etoposide treatment. No substantial increase in the number of cells undergoing apoptosis was found to occur in the 1 μ M etoposide treatment group, which suggests that increased expression of PARP, as well as its localization into the nucleus, would inhibit the induction of apoptosis in cells. Cells treated using 5 μ M etoposide were found to be undergoing apoptosis, as indicated by nuclear blebbing and membrane fragmentation. There was no detectable increase in PARP expression and PARP was found to be primarily localized in the cytoplasm of cells. This localization was not anticipated, but could potentially be due to the cleavage of PARP and its subsequent release of the apoptosis-inducing factor (AIF). AIF is released by PARP upon detection of irreparable damage, following which AIF then localizes to mitochondria to help facilitate cytochrome c release [51, 52]. However, it is currently not fully understood how PARP mediates the release of AIF [49]. Thus, further investigation would be required to determine if AIF release resulted in the apparent localization of PARP to the cytoplasm. The ability of PARP to spare cells from apoptosis, as well as its cleavage resulting in the release of AIF, provide reason for TAL1 to spare PARP from degradation.

Elucidating the relationship that exists between TAL1 and its potential modulation of PARP expression and function could possibly lead to the identification of novel targets for future treatments of T-ALL.

The outcome of patients with primary resistant or relapsed T-ALL remains poor, often requiring extensive and life-threatening chemotherapy [9]. The ability of TAL1 to mediate apoptotic resistance in T-ALL by potentially influencing death-associated proteins remains to be fully understood. The successful creation of an immunocytochemistry protocol in this study will serve to help further the understanding of which proteins are influenced by TAL1 expression. Further studies are warranted in order to confirm the present findings, and to investigate the influence of TAL1 in modulating the expression and function of other death-associated proteins such as BID, caspase-3, caspase-8 and NUR77. Until more effective chemotherapy treatments are created for the treatment of T-ALL, the need to identify potential TAL1 modulated targets will continue to be of importance to oncological research and warrant further investigation.

MATERIALS

- 1X RPMI 1640 + L-Glutamine (11875-093, Life-Technologies)
- 10% BGS (SH30541.03, Thermo-Fisher)
- Trypan Blue (T8154, Sigma)
- Etoposide (BML-GR307-0100, Enzo)
- Dimethyl Sulfoxide (BP231-100, Thermo-Fisher)
- Dual-Chamber Hemacytometer (1483, Hausser Scientific)
- 15 mL centrifuge tubes (05-539-1, Thermo-Fisher)
- 1X PBS (70013-032, Life-Technologies)
- 4% Paraformaldehyde (M134-500ML, Amresco)
- Triton X-100 (T8787-50ML, Sigma)
- 5% Goat Serum (SH30541.03, Thermo-Fisher)
- 0.5% BSA (0332-100G, Amresco)
- 1:600 Mouse anti- β -actin 1^o Antibody (#3700, Cell Signaling)
- 1:200 Rabbit anti-Bcl-X_L 1^o Antibody (#2764, Cell Signaling)
- 1:50 Rabbit anti-NF κ B/p65 1^o Antibody (#8242, Cell Signaling)
- 1:400 Rabbit anti-cleaved PARP 1^o Antibody (#5625, Cell Signaling)
- 1:1000 Anti-Mouse Alexa Fluor 488 2^o Antibody (#4408, Cell Signaling)
- 1:1000 Anti-Rabbit Alexa Fluor 488 2^o Antibody (#4412, Cell Signaling)
- ProLong Gold antifade with DAPI (P36935, Life-Technologies)

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