ECTOPIC EXPRESSION OF TAL-1 INCREASES RESISTANCE TO TNFα-INDUCED APOPTOSIS IN JURKAT CELLS VIA CHANGES IN THE NF-κB SIGNALING PATHWAY

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**Introduction**

The goal of this research was to ascertain the role of TAL-1 in resistance to drug-induced cell death. Approximately 25% of T-ALL patients show tumor-specific DNA rearrangements that result in the production of TAL-1 transcripts and polypeptides by their leukemic cells (1). These transcripts and polypeptides result in TAL-1 being ectopically expressed in T-ALL patients (2). T-ALL accounts for approximately 15% of all pediatric acute lymphoblastic leukemia cases and approximately 25% of all adult acute lymphoblastic leukemia cases. Furthermore, it is thought that ectopic expression of TAL-1 may correlate to poor response to chemotherapy (3).

Chemotherapeutic drugs kill rapidly dividing cells by inducing cell death. Any kind of unwanted change in a cell elicits an apoptotic response. Apoptosis is a normal process in which a programmed sequence of events leads to cell death (4). This process plays a pivotal role in developing and maintaining a healthy body by eliminating old, unnecessary, and unhealthy cells. Apoptosis is carried out by a family of cysteine proteases called caspases (5). There are two major pathways for caspase activation that have been identified: the receptor-mediated pathway and the mitochondrial-mediated pathway (6).
The mitochondrial-mediated pathway centers on the release of cytochrome c from mitochondria. Cytochrome c is normally found between the inner and outer membranes of mitochondria. In response to certain factors, cytochrome c is released and binds to Apaf-1, forming an apoptosome. Pro-caspase-9 will then bind to the apoptosome at Apaf-1 where it will be cleaved by autoproteolysis into active caspase-9. Active caspase-9, in turn, cleaves the downstream effector caspase-3 (5). This activates the caspase cascade that will induce cell death through cleavage of numerous cellular proteins.

This research will focus on the receptor-mediated pathway which begins with the TNFα family of cytokine receptors: Fas, TNF-R1, and TNF-R2. When bound by a cytokine, receptor dimerization occurs allowing the adaptor proteins FAS-associated death domain (FADD) or TNF receptor associated death domain (TRADD) to bind. These proteins, as stated by their name, contain a death domain (DD) that interacts with the DD on the cytoplasmic side of the receptor (7). These adaptor proteins also contain a death effector domain (DED) which allows them to interact with the death effector domains on other apoptotic proteins such as pro-caspase-8 and pro-caspase-10. When these pro-caspases are positioned close together, autoproteolysis or self-cleavage occurs. Activation of these initiator caspases, in turn, leads to the activation of caspase-3. This activates the caspase cascade that will induce cell death.

TNFα is a cytokine that functions by influencing the transcriptional activation and expression of a variety of molecules such as NF-κB and tumor necrosis factor receptor-associated factors (TRAF-1, TRAF-2). TNFα induces its
response through interactions with its receptors: TNF-R1 and TNF-R2. The TNF-R1 receptor is expressed on somatic cells, while the TNF-R2 receptor is typically expressed on immune cells (7). The TNF-R1 receptor has been widely studied, but the biological activity of TNF-R2 is not well understood. The mechanism behind which receptor is utilized is unclear. It is hypothesized that when TNF-R2 is bound by its ligand, the TNF-R2 signal may be passed to TNF-R1 to enhance signaling (8).

The molecular details involved in the NF-κB pathway have only recently been getting a lot of attention (9). The specific roles of different NF-kB subunits needs to be further explored, but the NF-kB pathway has been identified as a new target for cancer research. Chan and Lenardo (10) found that over expression of TNF-R2 in Jurkat cells helped overcome the resistance of this cell line to TNFα-induced cell death, decreased TRAF2 protein levels, and enhanced NF-kB activation. They recognized that cell death was controlled primarily by TNF-R1, but the TNF-R2 signal dramatically increased the magnitude and rate of caspase activation and cell death induced by TNF-R1.

Expanding on this research, Wicovsky et al. (11) found that both TNF-R1 and TNF-R2 were needed for optimal signal transduction in the cell. A model depicting this is shown in Figure 1. They showed that TRAF1 strongly interacted with TRAF2, a mediator of NF-kB activation. These TRAF1-TRAF2 complexes were co-recruited to both TNF-R1 and TNF-R2. TRAF2 associated with TNF-R2 to dramatically increase NF-kB activation. However, when a TRAF1-TRAF2 complex associated with TNF-R2, this activation of NF-kB was much more robust.
than with TRAF2 alone. They speculated that enhanced TNF-R2-induced activation of NF-kB was attributed to the formation of TRAF1-TRAF2 complexes that were not seen when TNF-R1 was activated without TNF-R2.

**Figure 1.** Model of TNF-R1-TNF-R2 cooperation and TRAF1-TRAF2 complex formation. Taken from Wivocsky et al. (11).
Additionally, Legarda-Addison et al. (12) found that both the receptor interacting protein-1 (RIP1) and NEMO (or IKKγ) protein played essential roles in mediating whether the TNF-R1 receptor initiated survival or apoptosis through the NF-κB pathway. Much like TNF-R1, RIP1 is a dual functioning molecule that can be pro-apoptotic or anti-apoptotic depending on its ubiquitination state (13). They showed that when RIP1 was ubiquitinated, TNFα-induced cell death was inhibited. However, when RIP1 was not ubiquitinated, it served as a pro-apoptotic signaling molecule through its association and subsequent activation of caspase-8. Further analysis identified NEMO as having the ability to interact with RIP1 (12). When NEMO was not present in the NF-κB pathway, RIP1 was free to associate with caspase-8 and initiate the caspase cascade. However, when NEMO was active, it associated with and sequestered RIP1 preventing it from interacting with caspase-8.

Thus, this research project set out to determine if ectopic expression of TAL-1 increases resistance to drug-induced cell death in cultured Jurkat cells via changes in the NF-κB signaling pathway. TAL-1 expression in the Jurkat cells was confirmed using flow cytometry. Jurkat cells expressing TAL-1 were split into an experimental group and a control group. The experimental group of Jurkat cells was treated individually or in combination with the cell death-inducing drug staurosporine and tumor necrosis factor. The control group of Jurkat cells remained untreated. Following treatments, Western blot analysis was performed on protein extracts to determine the expression levels of TAL-1, NF-κB, IKKγ, and TRAF-2. Changes in protein expression levels between the control and
experimental groups of Jurkat cells were assessed by densitometry. Flow cytometry was performed in order to ascertain how much cell death was occurring in the experimental group compared to the control group. Flow cytometry was also performed in order to ascertain caspases -3 and capsase-8 activity. Ultimately, the goal was to determine if drug-induced cell death was also influenced by the expression of these proteins involved in the NF-κB signaling pathway.
**Literature Review**

**Cell Cycle Control**

Normally, multiple signals are required to maintain proper cell growth (14). However, malignant transformation is driven by both intrinsic genomic changes and the response to extrinsic factors such as growth factors, cytokines, and chemicals (15). A cell undergoes numerous genetic changes as it transitions from its participation in normal homeostasis to malignancy. Several pathways have been found to be involved in malignant transformation, including those involved in cell cycle control and cell death.

The cell cycle is not a continuous process. Cell division is characterized by DNA replication and segregation into two separate cells (16). The cell cycle can be broadly divided into two stages: mitosis and interphase. Mitosis is further divided into prophase, metaphase, anaphase and telophase stages. Interphase is further divided into G1, S, and G2 stages. During interphase, the cell appears to only be growing in size. However, there are actually many processes occurring. During the G1 phase, the cell is preparing for DNA synthesis which will occur in the S phase. Then, during the G2 phase, the cell is preparing for mitosis. Before entering the G1 phase, cells can be forced into a quiescent state (G0)
where they are inactive. However, these cells can be stimulated to re-enter the cell cycle when needed.

Progression through G1, S, G2, and M phases of the cell cycle is controlled by a series of regulatory proteins called cyclins (15). These proteins bind to and form a complex with specific cyclin-dependent kinases (CDKs) which then target various proteins for phosphorylation. Kinases and phosphatases control CDK activity through differential phosphorylation. Regulation of CDK activity occurs in the form of inhibitors and cell cycle checkpoints.

The G1/S phase checkpoint depends upon two factors. First, this checkpoint relies on the inhibition of E2 promoter binding factor (E2F) transcription factors by the retinoblastoma (Rb) protein. The Rb gene is a tumor suppressor gene that typically provides a link between cell cycle machinery and transcription (16). S phase and mitotic cyclin-CDK complexes keep Rb phosphorylated so that it dissociates from the E2F factors allowing the cell to enter into the S phase (15). Second, this checkpoint relies on p53 (tumor suppressor protein) to control the expression of the CDK inhibitors (CDKI) p21 and p16. The p21 gene encodes a cyclin-dependent kinase inhibitor that will bind to, phosphorylate and inhibit all CDK-cyclin complexes (16). Phosphorylation of CDKIs prevents their activity and allows the cell to proceed through the S phase. The p16 protein is thought to bind CDK4 to prevent the cell from proceeding through the cell cycle. This process prevents inappropriate proliferation in normal cells. The G2/M phase checkpoint relies on phosphorylation-dependent inactivation of the CDC25 phosphatase by the checkpoint kinases Chk1 and 2.
Additionally, a checkpoint in the M phase will arrest mitosis if the spindles are not assembled correctly or if chromosomes are not correctly attached to the spindle. All of these regulators and inhibitors are essential to maintain proper cell cycle progression and homeostasis.

One such regulator of cell homeostasis is nuclear factor-kappa beta (NF-kB). NF-κB activation has been linked to signaling that controls cell cycle progression. More recently, it has been shown that tumor necrosis factor alpha (TNFα) and NF-kB family members play a role in carcinogenesis (9). Also, NF-kB has been found to control the expression of genes involved in the immune and inflammatory response, disease pathology and even cell death.

**Apoptosis and Caspases**

The most common response to any kind of unwanted change in a cell is apoptosis, or cell death. Apoptosis is a normal process in which a programmed sequence of events leads to cell death. Cells that undergo apoptosis portray certain characteristics including membrane blebbing, shrinkage, and chromatin condensation (4). Translocation of phosphatidylserine to the outer portion of the plasma membrane is also a specific characteristic observed during apoptosis. This process plays a crucial role in developing and maintaining a healthy body by eliminating old cells, unnecessary cells, and unhealthy cells. Eliminating old or unwanted cells is important during development, tissue remodeling, homeostasis, and immune responses. Our understanding of apoptosis and the mechanisms
involved came from genetic studies of the nematode *Caenorhabditis elegans* (17). A set of genes called cell death-defective (*CED*) were identified as regulators of the apoptotic process. These genes share functional homology with mammalian caspase genes.

Apoptosis is carried out by a family of cysteine proteases called caspases (5). Caspases cleave substrates such as poly ADP-ribose polymerase (PARP), nuclear lamins, DNA fragmentation factor (DFF), and Bcl-2 proteins at specific aspartate residues (17). All caspases are synthesized as inactive pro-enzymes (5). In their inactive form, caspases contain an N-terminal pro-domain and two catalytic domains. When the pro-domain is cleaved off at an aspartate residue, the caspase is activated. There is a generally accepted model of initiator caspase activation that relies on two important actions (18). First, autoproteolysis occurs between two pro-caspase forms at the linker segment between the pro-domain and the catalytic domains. Second, for true catalytic activity to occur, the two catalytic domains must associate to form a homodimer. This homodimer is responsible for cleaving and activating downstream caspases.

Based on their specific structure and when they are activated in the death pathway, caspases are classified as either initiator or effector caspases (17). Effector caspases are the proteases that cleave specific substrates in order to initiate the changes that are associated with cell death. Initiator caspases are responsible for cleaving and activating these effector caspases (18). Initiator caspases most often exist as monomers and have a long pro-domain, whereas, effector caspases most often exist as dimers and have a small pro-domain.
Initiator caspases include caspase-2, -8, -9, and -10 (17). Effector caspases include caspase-3, -6, and -7. Once activated, initiator caspases, in turn, activate other family members initiating what is called a caspase cascade. Initiator caspases are activated early in the death pathway and exercise a regulatory function by activating downstream effector caspases.

Caspase-8 has been identified as the most active initiator caspase. This initiator caspase is activated when a ligand binds to a death receptor (18). The pro-domain of this caspase contains a duplicate death effector domain (DED) allowing it to make multiple interactions. Proteolytic processing of caspase-8 has been shown to occur in chemotherapeutic drug-induced cell death, mitochondrial-mediated cell death, and receptor-mediated cell death. One study, using a Jurkat T cell line deficient in caspase-8, indicated resistance to cell death in these cells (19). This solidifies the importance of caspase-8 in the caspase cascade and cell death overall. Additionally, caspase-10 is activated when a ligand binds a death receptor (18). However, this initiator caspase has not been as extensively studied as caspase-8. Regardless of the pathway in which they are active, initiator caspases perform the same essential function.

Two major pathways for caspase activation have been identified: the receptor-mediated pathway which involves members of the TNF family of death receptors and the mitochondrial-mediated pathway which involves cytochrome c release from the mitochondria (6). Cross-communication between these two pathways can increase the speed and efficiency of the apoptotic process (17).
Mitochondrial-Mediated Pathway

The mitochondrial-mediated pathway begins with the release of cytochrome c from mitochondria. Normally found between the inner and outer membranes of mitochondria, cytochrome c will be released in response to apoptotic stimuli (20). The function of cytochrome c found in the cytosol is distinctly different from the function of cytochrome c found in mitochondria (21). Normally, cytochrome c functions in electron transport within the mitochondrial membrane. When cell death ensues and cytochrome c is released into the cytosol, its function changes to that of caspase activation. Once released, cytochrome c will bind to Apaf-1 and form what is called an apoptosome (22). This apoptosome serves to promote and initiate the caspase cascade where Apaf-1 serves as a docking site for the caspase cleaving reaction (17). Pro-caspase-9 binds to the apoptosome at Apaf-1, leading to autoproteolysis and activation. Once activated, pro-caspase-9, in turn, cleaves and activates the downstream effector caspase-3 (5). Caspase-3 then initiates cell death through cleavage of numerous target proteins. A diagram of the mitochondrial-mediated pathway is shown in Figure 2.

There are multiple agents that can play a role in the mitochondrial-mediated pathway. These are classified as activators, intermediates, and effectors, depending upon how they affect the apoptotic pathway. Activators are the stresses put upon the cell that will initially cause mitochondrial disruption. Examples include chemotherapeutic drugs, radiation, or cytokine withdrawal.
Figure 2. Mitochondrial-mediated pathway of cell death. Taken from the Cell Signaling Technology website.
Intermediates are the cell signaling molecules involved in the induction of cell death. These include second messengers such as JNK or JAK/STAT, as well as, oncoproteins such as p53, Myc, and Rb. Effectors are the components of the cell that directly affect disruption of mitochondria and include both the pro- and anti-apoptotic Bcl-2 family members.

There are a couple different theories about how cytochrome c is released from mitochondria. In one theory, a variety of signals trigger small channels on the inner mitochondrial membrane to open (21). This allows water and other contents to enter and cause swelling. Eventually, the swelling causes the outer mitochondrial membrane to burst, releasing cytochrome c. The second theory, and more widely accepted theory, employs pro-apoptotic members of the Bcl-2 family of proteins that act to create pores in the outer mitochondrial membrane. This allows the release of cytochrome c without affecting the function of the inner mitochondrial membrane; more specifically, no loss of membrane potential or physical swelling (6).

The Bcl-2 family of proteins plays a major role in the intrinsic apoptotic pathway and have been broadly classified into two groups. There are pro-apoptotic family members including Bcl-xs, Bax, Bak, Bid, Bad, Bim, Noxa, Mtd and Puma and also anti-apoptotic family members including Bcl-2, Bcl-xL, A1, and Mcl-1 (6). All of the proteins share one or more homologous Bcl-2 domains including BH1, BH2, BH3 and BH4. The BH1 and BH2 domains are found in all of the anti-apoptotic family members and the pro-apoptotic family members including Bax, Bak, and Mtd. These domains are involved in channel and pore
formation. The BH3 domain is found in all members of the Bcl-2 family and this
domain mediates dimerization. Finally, the BH4 domain is found mainly in anti-
apoptotic family members. This domain is important to protein-protein
interactions between members of the Bcl-2 family and additional proteins that
control progression through the cell cycle.

The most important function of the Bcl-2 family of proteins is to control the
integrity of the outer mitochondrial membrane (22). The Bcl-2 proteins work
together in response to cellular stress to induce or inhibit cell death through
dimerization with one another (6). Bax and Bak act at the mitochondrial
membrane to induce cell death. They associate via their BH3 domains and insert
themselves into the mitochondrial membrane via their BH1 or BH2 domains to
form pores (23). This permeabilizes the membrane and allows cytochrome c and
other intermembrane proteins to escape into the cytosol. Anti-apoptotic Bcl-2 and
Bcl-xL proteins exhibit some control over this process by associating with Bax
and Bak at the mitochondrial membrane to inhibit their activity (6). However, Bid,
which can be cleaved into tBid, promotes cell death by inhibiting Bcl-2, thus
activating Bax and Bak. Some pro-apoptotic Bcl-2 proteins, such as Bad and
Noxa, do not directly activate Bax and Bak at the mitochondrial membrane.
Instead, Bad and Noxa release proteins that are normally sequestered by anti-
apoptotic family members thus promoting membrane permeabilization (22).
Additionally, Bad can bind to Bcl-2 and Bcl-xL and prevent them from interacting
with Bax or Bak. This allows Bax and Bak to continue pore formation at the
mitochondrial membrane that allows for release of cytochrome c. Overall, the
anti-apoptotic family members have emerged as important inhibitors of the mitochondrial-mediated pathway of cell death and thus are common targets of therapeutic research.

**Receptor-Mediated Pathway**

The receptor-mediated pathway starts with the TNFα family of cytokine receptors including Fas, TNF-R1, and TNF-R2. When the receptor is bound by a cytokine, it is activated through dimerization which leads to the binding of adaptor proteins such as FADD or TRADD. These proteins, as is stated by their name, contain a DD that will interact with the DD on the cytoplasmic side of the receptor (29). These adaptor proteins also contain a death effector domain (DED). Once they bind to the receptor, the DED allows them to interact with the DED on other apoptotic proteins such as pro-caspase-8 and pro-caspase-10. When these pro-caspases are positioned close together, autoproteolysis or self-cleavage occurs. Activation of these initiator caspases, in turn, leads to the activation of caspase-3. A diagram of the receptor-mediated pathway is shown in Figure 3.

The TNF-R1 receptor is found on most somatic cells, while the TNF-R2 receptor is typically found only on cells of the immune system (29). TNF-R1 has been widely studied as to its function in cell death. TNF-R1 can trigger an apoptotic response through the activation of caspase-8 by interactions of the DEDs or a pro-survival response through the activation of the NF-κB pathway.
Figure 3. Receptor-mediated pathway of cell death. Taken from the Cell Signaling Technology website.
The mechanism behind which pathway is activated is unclear. The biological activity of TNF-R2 is much less well known. The TNF-R2 receptor has been shown to be active in T-lymphocytes and seems to play an auxiliary role in cellular responses.

Along with TNF-R1 and TNF-R2, the Fas receptor is also associated with the receptor-mediated pathway of cell death. When the Fas ligand binds the Fas receptor, the death effector protein FADD/Mort-1 is recruited to the receptor. Once FADD/Mort-1 binds to the receptor, pro-caspase-8 is recruited and cleaved by autoproteolysis into its active form. When released back into the cytosol, caspase-8 will cleave and activate caspase-3. The Fas receptor can also bind adaptor proteins that, in turn, activate cell death signal-regulating kinase 1 (ASK-1). ASK-1 is a critically important mitogen activated protein (MAP) kinases that acts upstream of p38 and c-Jun terminal kinase (JNK) (7). Once activated, ASK-1 will associate with tumor necrosis factor receptor-associated factor 2 (TRAF2) and this starts a series of phosphorylation events that concludes with the activation of JNK’s. JNKs are activated through the phosphorylation of tyrosine and threonine residues (29). Once activated, JNKs will translocate into the nucleus and phosphorylate transcription factors to enhance transcriptional activity. JNK can induce cell death by phosphorylating transcription factors such as c-Jun and p53 at their amino-terminal activation domains.

ASK-1 can also associated with TRAF2 to start a series of phosphorylation events that results in the release of free NF-κB from an inhibitor called inhibitor of kappa beta (IκB). Once NF-κB is released, it will migrate to the nucleus. Here, it
induces transcription of survival genes such as FLICE-like inhibitory protein (FLIP) and inhibitors of cell death proteins (IAPs). There are numerous IAPs that can associate with TNF receptors (c-IAP) or block the activity of caspases (XIAP). These proteins can directly bind to and inhibit the activity of caspase-3 and caspase-7 or associate with TRAFs to inhibit the activation of caspase-8 (29).

NF-κB is a transcription factor that plays a role in inflammation, cell death, and cell proliferation (7). A number of cell stresses such as viral infection or T- and B-cell activation can lead to the activation of NF-κB. Improper activation of the NF-κB pathway can lead to autoimmune diseases, viral infection, and cancer (15). The NF-κB family of transcription factors (NF-κB1, NF-κB2, RelA, RelB, and c-Rel) induce expression of additional anti-apoptotic factors which regulate the receptor-mediated pathway of cell death. However, NF-κB transcription factors are sequestered in the cytoplasm by inhibitory proteins of Ikβ (Ikβ-α, Ikβ-β, Ikβε, and Bcl-3). The central regulatory step in the NF-κB signaling pathway is the activation of the IKK complex. This complex is composed of two catalytic subunits: inhibitor of kappa beta kinase α (IKKα) and inhibitor of kappa beta kinase β (IKKβ) and a regulatory subunit called IKKγ or NF-κB essential modifier (NEMO) (29). The IKK complex is responsible for the phosphorylation and degradation of Ikβ resulting in the translocation of NF-κB to the nucleus. It has been shown that when NEMO is absent, the IKK complex cannot be activated (13). This blocks the NF-κB pathway leaving these transcription factors sequestered in the cytoplasm. When NF-κB is blocked, it cannot exhibit
transcriptional control over genes involved in immune and inflammatory response or cell proliferation.

Recently, it has been shown that both the receptor interacting protein-1 (RIP1) and NEMO protein play essential roles in mediating whether the TNF-R1 receptor initiates survival or cell death through the NF-kB pathway (12). Much like TNF-R1, RIP1 is a dual functioning molecule that can be pro-apoptotic or anti-apoptotic depending on its ubiquitination state (13). It has been shown that when RIP1 is ubiquitinated, TNFα-induced cell death is inhibited. However, when RIP1 is not ubiquitinated, it serves as a pro-apoptotic signaling molecule through its association and subsequent activation of caspase-8. Further analysis identified NEMO as having the ability to interact with RIP1 (12). When NEMO is absent, RIP1 is free to associate with caspase-8 and initiate the caspase cascade. However, when NEMO is active, it can associate with and sequester RIP1 thus preventing it from interacting with caspase-8. The molecular details involved in the NF-kB pathway have only recently been understood (9). The specific roles of different NF-kB subunits still needs to be explored further, but the NF-kB pathway has been identified as a target for new anti-cancer therapies through the use of NF-kB inhibitors.

**Tumor Necrosis Factor α**

TNFα is a cytokine that is part of the TNF family of ligands (6). The TNF family includes TNFα, TNFβ, Fas, and tumor necrosis factor-related cell death
inducing ligand (TRAIL). TRAIL has emerged as a drug of interest when inducing cell death through the receptor-mediated pathway (30). Much like other members of the TNF ligand family, TRAIL induces cell death by binding to its receptor and initiating a caspase cascade. Additionally, it has been determined that normal and cancerous cells have different sensitivities to TRAIL treatment. About 80% of available human tumor cell lines show some degree of sensitivity to TRAIL, while most normal human cell lines are resistant (30).

TNFα functions by influencing the transcriptional activation and expression of a variety of molecules including NF-κB, TRAF-1, TRAF-2, ASK-1, FADD, IKKα, IKKβ, IKKγ, JNK, and various MAP kinases. TNFα was identified as a product of lymphocytes and macrophages that was able to induce death in certain types of cells (31). Major cellular sources of TNFα production are cells involved with immune system responses: activated macrophages, lymphoid cells, natural killer (NK) cells, and neutrophils. Normally, TNFα targets the endothelium where it will stimulate the release of platelet-activating factor and other cytokines, thus allowing for increased vascular permeability. In neutrophils, TNFα activates the respiratory bursts and degranulation that leads to the release of cytotoxic components contained within the granules of the cell. Macrophages respond to TNFα by increasing their cytotoxic activity and production of cytokines. These processes are essential to a successful innate immune response.

Interest rose when TNFα was shown to have selective cytotoxic action on malignant cells (8). Isolated more than 15 years ago as a product of the immune system, TNFα was shown to kill tumor cells in vitro in mice (4). While TNFα
proved to be toxic in animal and human trials, it failed to fulfill expectations when it came to cancer treatment. TNFα induces its response through two different receptors: TNF-R1 and TNF-R2. These receptors are universally expressed on the majority of cells in the human body. For this reason, it is not surprising that almost all types of cells respond to TNFα. Cell death, antiviral activity, and activation of NF-κB are all mediated through contact of TNFα with TNF-R1, although association with TNF-R2 has been demonstrated in T-lymphocytes.

TNFα is a type two transmembrane protein found only on outer membranes with a signal-anchor sequence at its c-terminal domain (31). Being a type two transmembrane protein allows TNFα to self-assemble into a trimer via noncovalent bonds. This membrane bound form of the protein can be processed into a soluble trimeric protein. The reason behind trimer formation remains functionally unclear.

As previously stated, TNFα is produced in both a soluble (sTNFα) and membrane-bound (mTNFα) form. The two types seem to show different affinities for TNF receptors. TNF-R2 seems to preferentially bind mTNFα while TNF-R1 seems to preferentially bind sTNFα. One hypothesis, called ligand passing, states that TNF-R2 bound by mTNFα may pass the ligand over to TNF-R1 to enhance signaling. This hypothesis is supported by the off-rate of the two TNF receptors. Off rate refers to how long a ligand is bound to its given receptor. TNF-R2 has a high off-rate meaning it will bind mTNFα producing large amounts of TNFα on the surface of the cell and quickly release. The length of time the mTNFα ligand is associated with TNF-R2 (off-rate) is short. Instead of releasing
back into the cytosol, mTNFα is passed over to TNF-R1 in order to induce signaling not only through TNF-R2 but also TNF-R1. This cooperative signaling by both receptors leads to a much stronger response than what would be achieved alone.

**Staurosporine**

Phosphorylation of numerous target proteins is important to many cellular processes including cell signaling, cell cycle control and cell death (24). Many drugs have been developed that influence phosphorylation cascades in the hopes of returning regulation to the cell. One such drug is a compound called staurosporine. The compound was first discovered in 1977 when researchers were screening microbial alkaloids (25). The protein target of staurosporine was unknown until 1986 when it was discovered that this compound was a highly potent, albeit, non-specific protein kinase inhibitor. Today, it is accepted that staurosporine is a general kinase inhibitor (16). Staurosporine inhibits the activity of a protein kinase by competing with ATP at the binding domain on the kinase (25). Staurosporine can influence biological activities such as smooth muscle contraction, platelet aggregation, hyperplastic cell growth, and cell cycle control through inhibitory activity against protein kinase C (PKC), myosin light chain (MLC) kinase, CDKs, and numerous other protein kinases (16).

A major property of this drug is that it can drive virtually any mammalian cell into cell death (26). When cells are treated with staurosporine, it has been
determined that cytochrome c was released at every stage of the cell cycle (27) and cells displayed nuclear elongation, disruption of the nuclear membrane, and widespread fragmentation (28). Staurosporine can also induce mitochondrial damage resulting in the release of cytochrome c and the activation of caspase-9 (26). Furthermore, treatment with this drug induces G₁ and G₂ cell cycle arrest of normal cells, but will only induce G₂ cell cycle arrest of transformed cells. When this happens, cell death can then occur in the transformed cell.

Staurosporine is both highly hydrophobic and highly basic (25). This means that its biochemical activity may be dependent upon certain experimental conditions such as pH and exposure time. The effect of staurosporine on normal cells is thought to be concentration dependent (28). Depending upon the malignant cells, CDKs and cyclin-CDK complexes involved in the regulation of when a cell exits the G1 or G2 phase have different sensitivities to staurosporine. Lymphocytes and other malignant cells are more sensitive to staurosporine treatments at lower concentrations. While it is widely used, the primary mode of action of staurosporine in the induction of cell death is still unclear. It has been suggested that the mechanism through which staurosporine induces cell death is more complex than the caspase activation brought about by other anticancer drugs (26). It is likely that staurosporine induces cell death through multiple pathways and that inhibition of more than one kinase is responsible for its activity.
**TAL-1 and T-cell Acute Lymphoblastic Leukemia**

TAL-1 is a class two basic helix-loop-helix (bHLH) transcription factor that is required for normal hematopoiesis (32, 33). It has also been shown that TAL-1 acts cooperatively with LMO1 or LMO2. LMO1 and LMO2 encode LIM-only proteins that consist of two tandemly repeated LIM domains (34). The LIM domain is described as having a cysteine-rich zinc finger-like motif. The domain is found in certain transcription factors, kinases, and cytoskeletal proteins and is thought to mediate protein-protein interactions. LMO proteins are thought to be involved in transcriptional regulation, even though they do not have DNA binding activity. Overall, TAL-1 and LMO proteins have been shown to play a role in embryogenesis, vasculogenesis, upregulation of retinaldehyde dehydrogenase 2 (RALDH2), maturation of erythroid and megakaryocytic progenitors, and leukemogenesis (35, 36).

Ectopic expression of TAL-1 targets genes involved in the disruption of T-cell development and pathogenesis of T-cell acute lymphoblastic leukemia (T-ALL) (37). According to the current model of leukemogenesis, TAL-1 acts as a transcriptional repressor through its dimerization with class one bHLH proteins such as E2A and HEB (37). Heterodimerization with a class one bHLH member is required for TAL-1 function. The resultant heterodimers then bind to DNA in a sequence-specific manner at the E-box (38). The E-box is a DNA sequence that has been identified in the promoter regions of specific genes (37). It has been suggested that binding of TAL-1 to these bHLH proteins aids in the identification
and binding of the E-box sequence (38). When this happens, TAL-1 can interact with LMO1 or LMO2 which can then serve as mediators between TAL-1 and GATA-3. This blocks transcription of other bHLH factors such as E2A and HEB (39-41). Additionally, GATA-3 binding at the GATA site in the second intron of the RALDH2 gene promoter is blocked (42-44). Activation of RALDH2 stimulates production of retinoic acid which is necessary for embryonic limb, cardiac, and organ development (34). The production of retinoic acid has also been shown to inhibit cell death in T-cell acute lymphoblastic leukemia (T-ALL).

Even though TAL-1 plays a normal role in hematopoiesis, the manner in which it contributes to leukemogenesis is not completely understood. TAL-1 is not usually expressed in normal adult T lymphocytes, but ectopic expression is observed upon chromosomal translocation t(1;14)(p34;q11) (45). It also is not clear whether the TAL-1/bHLH protein heterodimers regulate transcription by binding directly to the E-box because no downstream targets have been identified. While the same has not been shown in humans, TAL-1 does not need to bind DNA in order to induce leukemia development in mice (45). It is thought that both TAL-1 and LMO proteins act synergistically, but how their ectopic expression impacts T-ALL is not known. It is suggested that alongside ectopic TAL-1 expression in the thymus, E2A inactivation may also contribute, at least in part, to the development of T-ALL. E2A-deficient mice produce a decreased number of thymocytes. More specifically, E2A-deficient mice have an increased number of thymocytes that have not yet committed to the T-cell lineage while the number of thymocytes that have committed to the T-cell lineage appears to be
almost non-existent. The result is a low number of mature and functional T-cells and a large number of self-reactive or abnormal T-cells being produced. Normally, self-reactive or abnormal T-cells will die by cell death. If cell death does not occur, T-ALL will develop. A better understanding of the mechanisms behind the developmental and transcriptional regulation by TAL-1 and LMO proteins could lead to a better understanding of their role in leukemogenesis.

T-ALL is part of a diverse group of lymphoid disorders that results from an overproduction of immature lymphocytes in the blood, bone marrow, or other organs (46). The biological and molecular cause of T-ALL aggressiveness and its poor response to traditional cancer therapies still remain elusive. The signal transduction pathways present in normal hematopoietic cells are aberrantly controlled in their malignant counterparts (46). Improper activation of these pathways by oncogenes has proven to be important in the pathogenesis of T-ALL. Therefore, factors involved in these pathways have increasingly become molecular targets of interest.

Much of what we know about T-ALL comes from studying immortalized T-lymphocyte cell lines such as the Jurkat cell line. These cells were originally established from the blood of a 14-year old boy with T-cell leukemia in the late 1970s (48). Their main use has been to study the susceptibility of malignant cells to chemotherapeutic drugs and radiation. T-ALL accounts for approximately 15% of all pediatric acute lymphoblastic leukemia cases and approximately 25% of all adult acute lymphoblastic leukemia cases (1). The root of T-ALL aggressiveness and its poor response to traditional cancer therapies still remain elusive. TAL-1,
an ectopically expressed transcription factor in 60% of T-ALL patients, could be linked to poor chemotherapy response (2, 3). Concurrently, 92% of adult patients with T-ALL show elevated NF-κB activation. The cause for this elevation is not known. Understanding how TAL-1 and NF-κB contribute to the development of T-ALL is important in the development of better therapies. It is hoped that the findings of this research will help in the advancement of more effective, targeted therapies for T-ALL.
Materials and Methods

Cell Culture and Drug Treatments

Jurkat cells were grown in RPMI 1640 (22400, Invitrogen) medium supplemented with 10% bovine growth serum (BGS) (SH30541.03, Thermo Scientific) and cultured in a humidified incubator at 37°C with 5% CO₂. Cells were treated either individually or in combination with the chemotherapeutic drug staurosporine (El-156, Enzo Life Sciences) and tumor necrosis factor alpha (TNFα) (300-01A, Peprotech). Treatments were performed with staurosporine at concentrations of 0.1 µM and 0.2 µM. Treatments were performed with TNFα at concentrations of 250 ng/mL and 500 ng/mL. Dual treatments with staurosporine and TNFα were performed at concentrations of 0.1 µM / 250 ng/mL and 0.2 µM / 500 ng/mL, respectively. All treatments were done over a 24 hr period.

Protein Extraction

To determine proteins present in cultured Jurkat cells, extracts from staurosporine- and/or TNFα-treated Jurkat cells were made using RIPA buffer (89900, Thermo Scientific). Cell suspensions were added to a 15 mL conical tube
and the cells were centrifuged at 1000 rpm for 5 min. The supernatant was discarded and cells were resuspended in 1 mL of cold 1X PBS (70013032, Invitrogen). Cells were again centrifuged for 5 min at 1000 rpm to pellet the cells. This process was repeated a second time. Then, 1 mL of RIPA buffer was added to each cell pellet and resuspended. The mixture was put into a 10cc syringe (309650, BD) and forced through a 21 gauge needle (305167, BD) in order to sheer the cells. The suspension was added to a 1.5 mL microfuge tube and rocked at 4°C for 30 min. The mixture was then centrifuged for 15 min at 10,000 rpm. The supernatant was transferred to a series of microfuge tubes and stored at -20°C until analyzed.

Protein Determination

Protein concentrations were determined using the Bio-Rad Bradford system. Protein samples were first thawed on ice. Next, a series of bovine serum albumin (BSA) (500-0007, Bio-Rad) protein standards and unknowns (Jurkat extracts) were prepared. The tubes were set up as follows: (1) 800 µl H₂O (blank), (2) 799 µl H₂O + 1 µl BSA/unknown protein, (3) 798 µl H₂O + 2 µl BSA/unknown protein, (4) 797 µl H₂O + 3 µl BSA/unknown protein, (5) 796 µl H₂O + 4 µl BSA/unknown protein, and 795 µl H₂O + 5 µl BSA/unknown protein. Then, the appropriate amounts of Jurkat protein extracts and Milli-Q water were added to 1.5 mL microfuge tubes. Two tubes for each sample were prepared in this fashion in order to perform the assay in duplicate. Once the protein and water
were added, 200 μl Bio-Rad developing reagent (500-0006, Bio-Rad) was added to all tubes. The contents were transferred to plastic cuvettes using transfer pipettes. Standard and unknown sample absorbancies were read at 595 nm. Standard values were used to generate a standard curve using Microsoft Excel software such that the protein concentration of each sample could be calculated using the slope equation derived from this curve.

**Western Blot Analysis**

To examine protein expression in cultured Jurkat cells, Western blot analysis was performed. Casting plates were aligned per manufacturer’s instructions and assembled into the casting stand. A 10% running gel was prepared as follows in a total of 10 mL: 4 mL water, 3.33 mL 30% acrylamide (VW1465-01, VWR)/0.8% bisacrylamide (BP171-100, Fisher Scientific) pH 8.8, 2.5 mL 4X Tris (BP152-1, Fisher Scientific)/SDS (VW1495-04, VWR), 50 μL 10% ammonium persulfate (APS) (UN1444, OmniPur), and 10 μL TEMED (UN2372, OmniPur). The mixture was then pipetted between the glass plates (about 1.5 lengths from top of smaller plate). A few drops of 1% SDS were added across the top of the running gel. The running gel was allowed to polymerize for 15-20 min.

The stacking gel was then prepared as follows in a total of 5 mL: 3.35 mL water, 650 μL 30% acrylamide/0.8% bisacrylamide pH 6.8, 1.25 mL 4X Tris/SDS, 50 μL 10% APS, and 5 μL TEMED. The fluid layer on top of the running gel was decanted and then the stacking gel was added on top of the running gel. A comb
was inserted between the plates and the stacking gel was allowed to polymerize for 15-20 min. During this time, 1000 mL of 1X SDS running buffer was made.

The standard curve created from the protein determination was used to determine how many micrograms of protein to add for each sample. One microliter of Lane Marker Sample Buffer (39000, Thermo Scientific) was added to each sample. The samples were heated for 5 min in boiling water and briefly centrifuged. The comb was removed and the wells were flushed with running buffer and then aspirated. Finally, the gel was placed into a Bio-Rad Mini-Protean III electrophoresis unit.

Samples, along with 1 µl of DyLight Infrared Protein Molecular Weight Marker (22859, Thermo Scientific), were loaded into the wells of the gel. Running buffer was added to top off each well and also used to fill both the inside and outside chambers. Careful attention was taken not to mix the buffer between the two chambers. The gel was run at 100V for 1.5 – 2 hrs.

When samples reached the bottom of the glass plates, the electrophoresis unit was turned off and disassembled. Semi-dry transfer was performed per the manufacturer’s instructions. The gel was transferred for 1 hr at 90 A. After this time, the transfer unit was disassembled. The nitrocellulose membrane (8484259, Whatman) was carefully placed into a plastic box containing 1X PBS. Three 1X PBS rinses were performed. After washing, the membrane was drained and then placed in a plastic container containing 3 mL Odyssey blocking buffer (927-40000, LI-COR). The container was then placed on a rocking platform shaker for 1 hr at 4ºC. Next, 3 µl of Tween-20 (BP337500, Thermo Scientific) and
3-6 µl of the primary antibody of interest (1:500 – 1:1000 dilution) (TAL-1: sc-22809, Santa Cruz Biotechnology; NF-κB: sc-1190, Santa Cruz Biotechnology; Actin: sc-1616, Santa Cruz Biotechnology; β-tubulin: 2128, Cell Signaling Technology; IKKγ: 6685, Cell Signaling Technology; TRAF-2: 4724, Cell Signaling Technology) were added to 3 mL Odyssey blocking buffer and mixed well. The membrane was placed back into the plastic container and allowed to rock overnight at 4ºC on a rocking platform shaker.

The next day, the membrane was placed in a plastic container and washed four times with a 1X PBS / 0.01% Tween-20 mixture. Each wash lasted 5 min. During the last wash, 3 µl of Tween-20 and 1.2 µL Alexa-Fluor 688 goat anti-rabbit secondary antibody (A21076, Molecular Probes) (1:25,000 dilution) were added to the Odyssey blocking buffer and mixed well. Once the last wash was poured off, the membrane was moved to the container with the Odyssey / 0.01% Tween-20 / secondary antibody mixture. The container was covered with foil and then rocked on a rocking platform shaker at room temperature for 45 – 60 min. After this time, the membrane was washed four times with a 1X PBS / 0.01% Tween-20 mixture. Each wash lasted 5 min. The membrane was then washed twice with plain 1X PBS. Each of these washes lasted 5 min. After these washes, the membrane was imaged on the LI-COR Odyssey Imaging System and image analyzed using Odyssey Imaging Software.
**Densitometry**

Densitometry was performed using Odyssey Imaging Software. A rectangle tool was used to draw boxes around bands. With the details view open, each box was selected in order to obtain quantification information. The data provided was exported into a Microsoft Excel spreadsheet. The data obtained for the protein of interest (TAL-1, NF-κB, IKKγ or TRAF-2) was normalized to the loading control (β-tubulin or actin). This information was used to quantify the bands and determine relative differences in intensity.

**Annexin V-FITC Cell death Assay**

The extent of cell death was determined using an Annexin V-FITC apoptosis detection kit (556547, BD Biosciences) and flow cytometry. Approximately 1x10^6 drug treated cells were centrifuged and the pellet was resuspended in 100 µl of 1X binding buffer. This mixture was then transferred to a 5 mL FACS tube. Then, 5 µl of Annexin V-FITC conjugate and 5 µl propidium iodide were added to the tube. Cells were gently vortexed and incubated in the dark at RT for 10 min. An additional 400 µl of 1X binding buffer was added and the mixture was then analyzed using an Accuri flow cytometer. Annexin V-FITC was used in conjunction with propidium iodide (PI) in order to identify both necrotic and apoptotic cell death. When cell death was measured over time, cells were tracked during all stages of cell death. Annexin V-FITC negative and PI
negative indicated no cell death, Annexin V-FITC positive and PI negative indicated apoptosis with membrane integrity still intact. Finally, Annexin V-FITC positive and PI positive indicated cell death through necrosis and apoptosis.

**Flow Cytometry Analysis of TAL-1 expression**

The presence of TAL-1 protein was determined using an Accuri flow cytometer. Approximately 1x10^6 cells were centrifuged and the pellet was resuspended in 1 mL of FACS buffer. This mixture was then transferred to a 5 mL FACS tube. Then, 1 mL of 0.5% Tween-20 / 1X PBS permeabilization buffer was added to the tube. The cells were centrifuged and the pellet resuspended in 100 µl of 0.5% Tween-20 / 1X PBS permeabilization buffer. The cells were incubated in the dark at RT for 15 min. Then, 2 mL of 0.5% Tween-20/1X PBS permeabilization buffer was added to the tube. The cells were centrifuged again and 10 µl of the TAL-1 primary antibody was added to the tube. Cells were then gently vortexed and incubated in the dark at RT for 30 min. After 30 min, the cells were centrifuged and 10 µl of the Zenon Alexa Fluor 488 rabbit labeling reagent (Z25302, Invitrogen) was added. Cells were gently vortexed and incubated in the dark at RT for 30 min. After 30 min, 2 mL FACS buffer was added to the tubes. After vortexing, the cells were centrifuged and the pellet resuspended in 600 µl of FACS and FIX buffer. The cells were then analyzed using the Accuri flow cytometer and the percent of intra-cellular TAL-1 plotted. Histograms were created using SigmaPlot software.
Flow Cytometry Analysis of Caspase-3 and Caspase-8 Activity

Caspase-3 and Caspase-8 activity was determined using an Accuri flow cytometer. Following 24 hr drug treatments, approximately $1 \times 10^6$ treated cells were centrifuged and the pellet was resuspended in 1 mL of FACS buffer. This mixture was then transferred to a 5 mL FACS tube. Then, 1 mL of 0.5% Tween-20 / 1X PBS permeabilization buffer was added to the tube. The cells were centrifuged and the pellet resuspended in 100 µl of 0.5% Tween-20 / 1X PBS permeabilization buffer. The cells were incubated in the dark at RT for 15 min. Then, 2 mL of 0.5% Tween-20/1X PBS permeabilization buffer was added to the tube. The cells were centrifuged again and 10 µl of either caspase-3 (sc-7890, Santa Cruz Biotechnology) or caspase-8 (9662, Cell Signaling Technology) primary antibody was added to the tube. Cells were then gently vortexed and incubated in the dark at RT for 30 min. After 30 min, the cells were centrifuged and 10 µl of the Zenon Alexa Fluor 488 rabbit labeling reagent was added. Cells were gently vortexed and incubated in the dark at RT for 30 min. After 30 min, 2 mL FACS buffer was added to the tubes. After vortexing, the cells were centrifuged and the pellet resuspended in 600 µl of FACS and FIX buffer. The cells were then analyzed using the Accuri flow cytometer and the percent of intracellular caspase-3 and caspase-8 plotted. Histograms were created using SigmaPlot software.
Statistical Analyses

Studies comparing data between two groups was assessed using a Student’s $t$-test. Studies comparing data between three or more groups was assessed using one-way ANOVA. All studies were performed in replicate and independent experiments repeated. Differences between groups were considered statistically significant when $p \leq 0.05$. 
Results

TAL-1 expression in cultured Jurkat cells.

To determine if TAL-1 was expressed in Jurkat cells, flow cytometry was performed. Jurkat cells were processed for intracellular staining and analyzed using an Accuri flow cytometer. Both the unstained control and the TAL-1 stained samples were analyzed in triplicate. To set initial parameters, an unstained control was analyzed (Figure 3). The healthy population of Jurkat cells was gated based on size and granularity. The percentage of the Jurkat population that was TAL-1 positive was only 0.23%.

Using the same gating parameters, Jurkat cells stained with a TAL-1 primary antibody and Zenon Alexa Fluor 488 rabbit labeling reagent were analyzed (Figure 4). The percentage of the Jurkat population that was TAL-1 positive was 94.9%. The observed increase was statistically significant. These results demonstrate that the Jurkat cells express TAL-1.
TAL-1 expression is higher in Jurkat cells treated with TNFα compared to untreated Jurkat cells, but lower in Jurkat cells treated with STS or a combination of TNFα/STS compared to untreated Jurkat cells.

To determine if treatments affect the expression of TAL-1, Western blot analysis was performed. It was determined that TAL-1 expression was different depending on the drug used for treatment (Figure 5). Jurkat cells were treated with TNFα, STS, or a TNFα/STS combination for 24 hours and then protein extraction performed as described previously. Following protein determination,
the protein extracts obtained from the treated Jurkats were processed and analyzed by Western blot analysis.

Figure 5. *TAL-1 expression varies with treatment.* Jurkat cells were either untreated or pre-treated (24 h) with TNFα (250 ng/mL or 500 ng/mL), STS (0.1 μM or 0.2 μM), or a TNFα and STS combination (0.1 μM STS + 250 ng/mL TNFα or 0.2 μM STS + 500 ng/mL TNFα). Proteins were extracted from the treated Jurkat cells and Western blot analysis was performed. The Western blot was probed with TAL-1 antibody then with Alexa Fluor 688 anti-rabbit secondary antibody. Fluorescence was detected using an Odyssey Infrared Imaging System. Band intensity measurements and background corrections were performed using Odyssey Imaging software.
When the Jurkat cells were treated with TNFα alone, TAL-1 expression was higher as compared to untreated Jurkat cells. The 250 ng/mL TNFα treated sample had a densitometry value of 4.85 and the 500 ng/mL TNFα treated sample had a densitometry value of 5.20 (Table 1). The untreated Jurkat sample had a densitometry value of 1.97 (Table 1). Thus, TAL-1 expression in Jurkat cells increased approximately 60% when treated with TNFα compared to the untreated control. Furthermore, treatment with a lower concentration of TNFα (250 ng/mL) increased TAL-1 expression almost as much as treatment with a higher concentration of TNFα (500 ng/mL). This is demonstrated through densitometry values (Table 1).

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>TAL-1 Intensity</th>
<th>Actin Intensity</th>
<th>Normalized Densitometry Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>4578.81</td>
<td>2318.44</td>
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<td>0.1 μM STS</td>
<td>1071.46</td>
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<td>0.2 μM STS</td>
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<td>1288.12</td>
<td>0.94</td>
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<td>250 ng/mL TNFα</td>
<td>7507.56</td>
<td>1549.45</td>
<td>4.85</td>
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<tr>
<td>500 ng/mL TNFα</td>
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<td>1452.12</td>
<td>5.20</td>
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<td>0.1 μM STS + 250 ng/mL TNFα</td>
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<td>1.01</td>
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<td>0.1 μM STS + 250 ng/mL TNFα</td>
<td>1769.15</td>
<td>2661.42</td>
<td>0.66</td>
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**Table 1.** *Densitometry evaluation of TAL-1 expression after various treatments.* Following Western blot analysis using an Odyssey Infrared Imaging System, band intensities were determined using Odyssey software. Intensity values obtained for TAL-1 were divided by intensity values obtained for actin to yield the normalized densitometry value.
When the Jurkat cells were treated with STS alone, TAL-1 expression was lower compared to untreated Jurkat cells. The 0.1 μM STS treated sample had a densitometry value of 0.77 and the 0.2 μM STS treated sample had a densitometry value of 0.94 (Table 1). The untreated Jurkat sample had a densitometry value of 1.97 (Table 1). Thus, TAL-1 expression in Jurkat cells decreased approximately 60% when treated with a lower concentration of STS (0.1 μM) compared to untreated controls. TAL-1 expression in Jurkat cells decreased approximately 50% when treated with a higher concentration of STS (0.2 μM) compared to controls. This demonstrated that treatments with a lower concentration of STS (0.1 μM) decreased TAL-1 expression about 10% more than treatment with a higher concentration of STS (0.2 μM).

When Jurkat cells were treated with a TNFα/STS combination, TAL-1 expression was lower compared to untreated Jurkat cells. The 0.1 μM STS and 250 ng/mL TNFα treated sample had a densitometry value of 1.01 and the 0.2 μM STS and 500 ng/mL TNFα treated sample had a densitometry value of 0.66 (Table 1). The untreated Jurkat sample had a densitometry value of 1.97 (Table 1). Thus, TAL-1 expression in Jurkat cells decreased approximately 50% when treated with lower concentrations of STS and TNFα (0.1 μM STS and 250 ng/mL TNFα) compared to untreated controls. This also indicated that TAL-1 expression in Jurkat cells decreased approximately 70% when treated with higher concentrations of STS and TNFα (0.2 μM STS and 500 ng/mL TNFα) compared to untreated controls. This demonstrated that dual treatment with higher concentrations of STS and TNFα (0.2 μM STS and 500 ng/mL TNFα) decreased
TAL-1 expression 20% more than a dual treatment with lower concentrations of STS and TNFα (0.1 µM STS and 250 ng/mL TNFα).

Overall, treatments with TNFα increased expression of TAL-1 while treatments with STS alone and STS/TNF combinations decreased expression of TAL-1. Treatment with TNFα increased TAL-1 expression (50%) similarly to how treatment with STS alone (50 – 60%) and STS/TNF combinations (50-70%) decreased TAL-1 expression.

**NF-κB expression is higher in Jurkat cells treated with TNFα compared to untreated Jurkat cells, but lower in Jurkat cells treated with STS or a combination of TNFα/STS compared to untreated Jurkat cells.**

To determine if treatments affect the expression of NF-κB, Western blot analysis was performed. It was determined that NF-κB expression differed depending on the drug used for treatment (Figure 6). Jurkat cells were treated with TNFα, STS, or a TNFα/STS combination for 24 hours and then protein extraction performed as described previously. Following protein determination, the protein extracts obtained from the treated Jurkats were processed and analyzed by Western blot analysis.

When the Jurkat cells were treated with TNFα alone, NF-κB expression was higher compared to untreated Jurkat cells. For the active p50 NF-κB subunit, the 250 ng/mL TNFα treated sample had a densitometry value of 0.24 and the 500 ng/mL TNFα treated sample had a densitometry value of 0.25 (Table 2). The
untreated Jurkat sample had a densitometry value of 0.12. For the inactive p100 NF-κB subunit, the 250 ng/mL TNFα treated sample had a densitometry value of 0.11 and the 500 ng/mL TNFα treated sample had a densitometry value of 0.12 (Table 2). The untreated Jurkat sample had a densitometry value of 0.05. Thus,

Figure 6. **NF-κB expression varies with treatment.** Jurkat cells were either untreated or pre-treated (24 h) with TNFα (250 ng/mL or 500 ng/mL), STS (0.1 μM or 0.2 μM), or a TNFα and STS combination (0.1 μM STS + 250 ng/mL TNFα or 0.2 μM STS + 500 ng/mL TNFα). Proteins were extracted from the treated Jurkat cells and Western blot analysis was performed. The Western blot was probed with NF-κB antibody then with Alexa Fluor 688 anti-rabbit secondary antibody. Fluorescence was detected using an Odyssey Infrared Imaging System. Band intensity and background corrections were performed using Odyssey Imaging software.
expression of either the inactive or active forms of NF-κB increased approximately 50% when treated with either concentrations of TNFα as compared to untreated controls. In this case, TNFα concentration did not affect NF-κB expression.

When Jurkat cells were treated with STS alone, expression of both forms of NF-κB was higher compared to untreated Jurkat cells. For the active p50 NF-κB subunit, the 0.1 μM STS treated sample had a densitometry value of 0.17 and the 0.2 μM STS treated sample had a densitometry value of 0.14 (Table 2). The untreated Jurkat sample had a densitometry value of 0.12. For the inactive p100 NF-κB subunit, the 0.1 μM STS treated sample had a densitometry value of 0.08 and the 0.2 μM STS treated sample had a densitometry value of 0.06 (Table 2). The untreated Jurkat sample had a densitometry value of 0.05. Thus, active p50 NF-κB expression in Jurkat cells increased approximately 30% when treated with a lower concentrations of STS (0.1 μM) compared to untreated controls. Active p50 NF-κB expression in Jurkat cells increased approximately 15% when treated with a higher concentrations of STS (0.2 μM) compared to untreated controls. This demonstrated that treatment with a higher concentration of STS (0.2 μM) increase NF-κB expression 15% more than treatment with a lower concentration of STS (0.1 μM).

When the Jurkat cells were treated with a TNFα/STS combination, NF-κB expression was higher compared to untreated Jurkat cells. For the active p50 NF-κB subunit, the 0.1 μM STS and 250 ng/mL TNFα treated sample had a densitometry value of 0.20 and the 0.2 μM STS and 500 ng/mL TNFα treated
sample had a densitometry value of 0.16 (Table 2). The untreated Jurkat sample had a densitometry value of 0.12. For the inactive p100 NF-κB subunit, the 0.1 μM STS and 250 ng/mL TNFα treated sample had a densitometry value of 0.09 and the 0.2 μM STS and 500 ng/mL TNFα treated sample had a densitometry value of 0.07 (Table 2). The untreated Jurkat sample had a densitometry value of

<table>
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<th>NF-κB Intensity</th>
<th>Actin Intensity</th>
<th>Normalized Densitometry Value</th>
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Table 2. Densitometry evaluation of NF-κB expression after various treatments. Following Western blot analysis using an Odyssey Infrared Imaging System, band intensities were determined using Odyssey software. Intensity values obtained for NF-κB were divided by intensity values obtained for actin to yield the normalized densitometry value.
0.05. Thus, active p50 NF-κB expression in Jurkat cells increased approximately 40% when cells were treated with lower concentrations of STS and TNFα (0.1 μM STS and 250 ng/mL TNFα) compared to untreated controls. Active p50 NF-κB expression in Jurkat cells increased approximately 30% when cells were treated with higher concentrations of STS and TNFα (0.2 μM STS and 500 ng/mL TNFα) compared to untreated controls.

The densitometry value obtained for the band in lane seven, which corresponds to the 0.1uM STS and 250ng/mL TNFα dual treated sample, was This demonstrates that dual treatment with lower concentrations of STS and TNFα (0.1 μM STS and 250 ng/mL TNFα) increased NF-κB expression 10% more than dual treatment with higher concentrations of STS and TNFα (0.2 μM STS and 500 ng/mL TNFα).

Overall, treatments with TNFα alone, STS alone and STS/TNF combinations increased expression of NF-κB. Treatment with TNFα increased NF-κB expression (50%) more than treatments with STS alone (15 – 30%) or STS/TNF combinations decreased TAL-1 expression (10 – 30%).

**TRAF-2 expression is higher in Jurkat cells treated with TNFα compared to untreated Jurkat cells, but lower in Jurkat cells treated with STS or a combination of TNFα/STS compared to untreated Jurkat cells.**

To determine if treatments affect the expression of TRAF-2, Western blot analysis was performed. It was determined that TRAF-2 expression varied
depending on the drug used for treatment (Figure 7). Jurkat cells were treated with TNFα, STS, or a TNFα/STS combination for 24 hours and then protein extraction performed as described previously. Following protein determination, the protein extracts obtained from the treated Jurkats were processed and analyzed by Western blot analysis.

When Jurkat cells were treated with STS alone or a TNFα/STS combination, TRAF-2 expression was not observed (no band). When Jurkat cells were treated with TNFα alone, TRAF-2 expression was higher compared to untreated Jurkat cells. The 250 ng/mL TNFα treated sample had a densitometry value of 0.022 and the 500 ng/mL TNFα treated sample had a densitometry value of 0.019 (Table 3). The untreated Jurkat sample had a densitometry value of 0.017. Thus, TRAF-2 expression in Jurkat cells increased approximately 20% when treated with the lower concentration of TNFα (250 ng/mL) compared to untreated

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>TRAF-2 Intensity</th>
<th>Actin Intensity</th>
<th>Normalized Densitometry Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>617.04</td>
<td>36892.02</td>
<td>0.017</td>
</tr>
<tr>
<td>250 ng/mL TNFα</td>
<td>664.56</td>
<td>30743.53</td>
<td>0.022</td>
</tr>
<tr>
<td>500 ng/mL TNFα</td>
<td>716.72</td>
<td>37241.12</td>
<td>0.019</td>
</tr>
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</table>

Table 3. Densitometry evaluation of TRAF-2 expression after various treatments. Following Western blot analysis using an Odyssey Infrared Imaging System, band intensities were determined using Odyssey software. Intensity values obtained for TRAF-2 were divided by intensity values obtained for actin to yield the normalized densitometry value.
Figure 7. *TRAF-2 expression varies with treatment.* Jurkat cells were either untreated or pre-treated (24 h) with TNFα (250 ng/mL or 500 ng/mL), STS (0.1 μM or 0.2 μM), or a TNFα and STS combination (0.1 μM STS + 250 ng/mL TNFα or 0.2 μM STS + 500 ng/mL TNFα). Proteins were extracted from the treated Jurkat cells and Western blot analysis was performed. The Western blot was probed with TRAF-2 antibody then with Alexa Fluor 688 anti-rabbit secondary antibody. Fluorescence was detected using an Odyssey Infrared Imaging System. Band intensity and background corrections were performed using Odyssey Imaging software.
controls. TRAF-2 expression in Jurkat cells only increased approximately 10% when treated with the higher concentration of TNFα (500 ng/mL) compared to untreated controls. Treatment with a lower concentration of TNFα (250 ng/mL) increased TRAF-2 expression 10% more than treatment with a higher concentration of TNFα (500 ng/mL).

**IKKγ expression is higher in Jurkat cells treated with TNFα compared to untreated Jurkat cells, but lower in Jurkat cells treated with STS or a combination of TNFα/STS compared to untreated Jurkat cells.**

To determine if drug treatments affect the expression of IKKγ, Western blot analysis was performed. It was determined that IKKγ expression was different depending on the drug used for treatment (Figure 8). Jurkat cells were treated with TNFα, STS, or a TNFα/STS combination for 24 hours and then protein extraction performed as described previously. Following protein determination, the protein extracts obtained from the treated Jurkats were processed and analyzed by Western blot analysis.

When Jurkat cells were treated with TNFα alone, IKKγ expression was higher compared to untreated Jurkat cells. The 250 ng/mL TNFα treated sample had a densitometry value of 0.11 and the 500 ng/mL TNFα treated sample had a densitometry value of 0.10 (Table 4). The untreated Jurkat sample had a densitometry value of 0.08. Thus, IKKγ expression in Jurkat cells increased approximately 30% when treated with a lower concentration of TNFα (250 ng/mL).
**Figure 8.** *IKKγ* expression varies with treatment. Jurkat cells were either untreated or pre-treated (24 h) with TNFα (250 ng/mL or 500 ng/mL), STS (0.1 μM or 0.2 μM), or a TNFα and STS combination (0.1 μM STS + 250 ng/mL TNFα or 0.2 μM STS + 500 ng/mL TNFα). Proteins were extracted from the treated Jurkat cells and Western blot analysis was performed. The Western blot was probed with *IKKγ* antibody then with Alexa Fluor 688 anti-rabbit secondary antibody. Fluorescence was detected using an Odyssey Infrared Imaging System. Band intensity and background corrections were performed using Odyssey Imaging software.
compared to untreated controls. In comparison, IKKγ expression in Jurkat cells increased approximately 20% when treated with a higher concentration of TNFα (500 ng/mL) compared to untreated controls. This demonstrates that treatment with a lower concentration of TNFα (250 ng/mL) increased IKKγ expression 10% more than treatment with a higher concentration of TNFα (500 ng/mL).

When the Jurkat cells were treated with STS alone, IKKγ expression was lower compared to untreated Jurkat cells. The 0.1 μM STS treated sample had a densitometry value of 0.06 and the 0.2 μM STS treated sample had a densitometry value of 0.06 (Table 4). The untreated Jurkat sample had a

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>IKKγ Intensity</th>
<th>Actin Intensity</th>
<th>Normalized Densitometry Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>3098.96</td>
<td>37732.28</td>
<td>0.08</td>
</tr>
<tr>
<td>0.1 μM STS</td>
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<td>33862.49</td>
<td>0.06</td>
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<tr>
<td>0.2 μM STS</td>
<td>1636</td>
<td>26438.4</td>
<td>0.06</td>
</tr>
<tr>
<td>250 ng/mL TNFα</td>
<td>4897.82</td>
<td>43597.18</td>
<td>0.11</td>
</tr>
<tr>
<td>500 ng/mL TNFα</td>
<td>4234.74</td>
<td>41481.26</td>
<td>0.10</td>
</tr>
<tr>
<td>0.1 μM STS + 250 ng/mL TNFα</td>
<td>2863.23</td>
<td>40873.74</td>
<td>0.07</td>
</tr>
<tr>
<td>0.1 μM STS + 250 ng/mL TNFα</td>
<td>2379.05</td>
<td>36578.9</td>
<td>0.07</td>
</tr>
</tbody>
</table>

**Table 4. Densitometry evaluation of IKKγ expression after various treatments.** Following Western blot analysis using an Odyssey Infrared Imaging System, band intensities were determined using Odyssey software. Intensity values obtained for IKKγ were divided by intensity values obtained for actin to yield the normalized densitometry value.
densitometry value of 0.08. Thus, IKKγ expression in Jurkat cells decreased
approximately 25% when treated with either concentration of STS compared to
untreated controls. This demonstrates that treatment with a lower concentration
of STS (0.1 μM) decreased IKKγ expression similarly to treatment with a higher
concentration of STS (0.2 μM).

When Jurkat cells were treated with a TNFα/STS combination, IKKγ
expression was lower compared to untreated Jurkat cells. The 0.1 μM STS and
250 ng/mL TNFα treated sample had a densitometry value of 0.07 and the 0.2
μM STS and 500 ng/mL TNFα treated sample had a densitometry value of 0.07
(Table 4). The untreated Jurkat sample had a densitometry value of 0.08. Thus,
IKKγ expression in Jurkat cells decreased approximately 15% when treated with
combinations of STS and TNFα compared to untreated controls. This
demonstrated that a dual treatment with lower concentrations of STS and TNFα
(0.1 μM STS and 250 ng/mL TNFα) decreased IKKγ expression similarly to a
dual treatment with higher concentrations of STS and TNFα (0.2 μM STS and
500 ng/mL TNFα).

Overall, treatments with TNFα alone increased IKKγ expression while
treatments with STS alone or STS/TNF combinations decreased IKKγ
expression. Treatment with TNFα increased IKKγ expression (20 – 30%) similarly
to how treatment with STS alone (25%) and STS/TNF combinations (15%)
decreased TAL-1 expression.
Cell death was higher in Jurkat cells treated with STS or a combination of TNFα/STS compared to Jurkat cells treated with TNFα.

The percentage of cells undergoing cell death following treatments was determined using an Annexin V-FITC apoptosis detection kit. It was determined that cell death was much higher when Jurkat cells were treated with STS or a TNFα/STS combination compared to Jurkat cells treated with only TNFα. To set the initial parameters, unstained and untreated Jurkat cells were analyzed (Figure 9).

**Figure 9. Untreated, unstained Jurkat cells show no cell death.** Untreated Jurkat cells were processed for surface staining and flow cytometry was performed. Staining was not performed on these Jurkat cells as they served as a gating parameter. Peaks represent the unstained and untreated Jurkat cell population. **Panel A.** Cells were analyzed using an FL2 laser and fluorescence detected using an Accuri flow cytometer. Gating was performed using Cflow Plus software. **Panel B.** Cells were analyzed using an FL1 laser. Fluorescence was detected and gating performed as described in Panel A.
A healthy population of Jurkat cells was gated based on size and granularity (not shown). The Cflow Plus software determined the percentage of the population that fell within the positive gate. The percentage of unstained and untreated Jurkat cells that were Annexin V or PI positive was only 0.05%.

Using the same gating parameters, untreated Jurkat cells were stained with either PI or Annexin-V and analyzed. PI stained, untreated Jurkat cells were analyzed (Figure 10A). The Cflow Plus software determined the percentage of the

![Graph A](image)

**Figure 10.** Untreated, stained Jurkat cells indicate cell death. Untreated Jurkat cells were processed for surface staining and flow cytometry was performed. **Panel A.** Cells were analyzed using an FL2 laser. The black, blue and red peaks represent unstained and untreated Jurkat cells. The green, pink and yellow peaks represent the untreated Jurkat cells stained with propidium iodine. Fluorescence was detected using an Accuri flow cytometer. Gating was performed using Cflow Plus software. **Panel B.** Cells were analyzed using an FL1 laser. The black, blue and red peaks represent unstained and untreated Jurkat cells. The green, pink and yellow peaks represent the untreated Jurkat cells stained with Annexin-V. Fluorescence was detected and gating performed as described for Panel A.
population that fell within the positive gate. A shift in the peak population indicates apoptosis is occurring. The percentage of cells that were PI positive was 10.3%. This means that 89.9% of the cells were not PI positive. Annexin-V stained, untreated Jurkat cells were also analyzed (Figure10B). The percentage of cells that were Annexin-V positive was 23.2%. This means that 64.5% of the cells were not Annexin-V positive.

As a positive control, ethanol treated Jurkat cells were stained with either PI or Annexin-V and analyzed. PI stained, ethanol treated Jurkat cells were analyzed (Figure 11A). The Cflow Plus software determined the percentage of

![Figure 11. Ethanol treated, stained Jurkat cells indicate cell death.](image)

**Figure 11. Ethanol treated, stained Jurkat cells indicate cell death.** Jurkat cells were pre-treated (1 min) with ethanol, processed for surface staining and flow cytometry was performed. **Panel A.** Cells were analyzed using an FL2 laser. The green, pink and yellow peaks represent unstained and untreated Jurkat cells. The black, blue and red peaks represent the ethanol Jurkat cells stained with propidium iodine. Fluorescence was detected using an Accuri flow cytometer. Gating was performed using Cflow Plus software. **Panel B.** Cells were analyzed using an FL1 laser. The green, pink and yellow peaks represent unstained and untreated Jurkat cells. The black, blue and red peaks represent the ethanol Jurkat cells stained with Annexin-V. Fluorescence was detected and gating performed as described for Panel A.
the population that fell within the positive gate. The percentage of cells that were PI positive was 96.8%. This means that 3.2% of the cells were not PI positive.

Annexin-V stained, ethanol treated Jurkat cells were also analyzed (Figure 11B). The percentage of cells that were Annexin-V positive was 94.9% corresponding to cell death. This means that 5.1% of the cells were not Annexin-V positive.

Using the positive and negative controls as parameters for what the treated samples should look like, Jurkat cells treated with TNFα, STS, or a combination of TNFα/STS were analyzed. Jurkat cells treated with 0.1 μM STS were stained with both PI and Annexin-V and analyzed (Figure 12). The Cflow

**Figure 12.** 0.1 μM STS treated, stained Jurkat cells indicate cell death. Jurkat cells were pre-treated (24 h) with STS (0.1 μM), processed for surface staining with both FITC and PI and flow cytometry was performed. Panel A. Cells were analyzed using an FL1 laser. The black, pink and red peaks represent unstained and untreated Jurkat cells. The blue, green and yellow peaks represent the 0.1 μM STS treated Jurkat cells stained with Annexin-V. Fluorescence was detected using an Accuri flow cytometer. Gating was performed using Cflow Plus software. Panel B. Cells were analyzed using an FL2 laser. The black, blue and red peaks represent unstained and untreated Jurkat cells. The green, pink and yellow peaks represent the 0.1 μM STS treated Jurkat cells stained with propidium iodine. Fluorescence was detected and gating performed as described for Panel A.
Plus software determined the percentage of the population that fell within the positive gate. A shift in the peak population indicates apoptosis is occurring. The percentage of cells that were PI positive was 0.7% and the percentage of cells that were Annexin-V positive was 21.9%, indicating early stages of apoptosis. Also, the percentage of cells that were both Annexin-V and PI positive was 77.4%, indicating late stages of apoptosis or death.

Jurkat cells treated with 0.2 µM STS were stained with both PI and Annexin-V and analyzed (Figure 13). The Cflow Plus software determined the

**Figure 13.** 0.2 µM STS treated, stained Jurkat cells indicate cell death. Jurkat cells were pre-treated (24 h) with STS (0.2 µM), processed for surface staining with both FITC and PI and flow cytometry performed. **Panel A.** Cells were analyzed using an FL1 laser. The black, blue and red peaks represent unstained and untreated Jurkat cells. The blue, green and yellow peaks represent the 0.2 µM STS treated Jurkat cells stained with Annexin-V. Fluorescence was detected using an Accuri flow cytometer. Gating was performed using Cflow Plus software. **Panel B.** Cells were analyzed using an FL2 laser. The black, blue and red peaks represent unstained and untreated Jurkat cells. The green, pink and yellow peaks represent the 0.2 µM STS treated Jurkat cells stained with propidium iodine. Fluorescence was detected and gating performed as described for Panel A.
percentage of the population that fell within the positive gate. A shift in the peak population indicates apoptosis is occurring. The percentage of cells that were PI positive was 0.2% and the percentage of cells that were Annexin-V positive was 22% indicating early stages of apoptosis. Also, the percentage of cells that were both Annexin-V and PI positive was 77.7% indicating late stages of apoptosis or death.

Jurkat cells treated with 250 ng/mL TNFα were stained with both PI and Annexin-V and analyzed (Figure 14). The Cflow Plus software determined the

![Figure 14](image.png)

**Figure 14.** 250 ng/mL TNFα treated, stained Jurkat cells indicate cell death. Jurkat cells were pre-treated (24 h) with TNFα (250 ng/mL), processed for surface staining with both FITC and PI and flow cytometry performed. **Panel A.** Cells were analyzed using an FL1 laser. The black, blue and red peaks represent unstained and untreated Jurkat cells. The blue, green and yellow peaks represent the 250 ng/mL TNFα treated Jurkat cells stained with Annexin-V. Fluorescence was detected using an Accuri flow cytometer. Gating was performed using Cflow Plus software. **Panel B.** Cells were analyzed using an FL2 laser. The black, blue and red peaks represent unstained and untreated Jurkat cells. The green, pink and yellow peaks represent the 250 ng/mL TNFα treated Jurkat cells stained with propidium iodine. Fluorescence was detected and gating performed as described for Panel A.
percentage of the population that fell within the positive gate. A shift in the peak population indicates apoptosis is occurring. The percentage of cells that were PI positive was 0.15% and the percentage of cells that were Annexin-V positive was 23.3%, indicating early stages of apoptosis. Also, the percentage of cells that were both Annexin-V and PI positive was 17.5%, indicating late stages of apoptosis or death.

Next, Jurkat cells treated with 500 ng/mL TNFα were stained with both PI and Annexin-V and analyzed (Figure 15). The Cflow Plus software determined

![Figure 15](image)

**Figure 15.** 500 ng/mL TNFα treated, stained Jurkat cells indicate cell death. Jurkat cells were pre-treated (24 h) with TNFα (500 ng/mL), processed for surface staining with both FITC and PI and flow cytometry performed. **Panel A.** Cells were analyzed using an FL1 laser. The black, blue and red peaks represent unstained and untreated Jurkat cells. The blue, green and yellow peaks represent the 500 ng/mL TNFα treated Jurkat cells stained with Annexin-V. Fluorescence was detected using an Accuri flow cytometer. Gating was performed using Cflow Plus software. **Panel B.** Cells were analyzed using an FL2 laser. The black, blue and red peaks represent unstained and untreated Jurkat cells. The green, pink and yellow peaks represent the 500 ng/mL TNFα treated Jurkat cells stained with propidium iodine. Fluorescence was detected and gating performed as described for Panel A.
the percentage of the population that fell within the positive gate. The percentage of cells that were PI positive was 0.25% and the percentage of cells that were Annexin-V positive was 26.5%, indicating early stages of apoptosis. Also, the percentage of cells that were both Annexin-V and PI positive was 14.2%, indicating late stages of apoptosis or death.

Dual TNFα/STS treated Jurkat cells were analyzed. Jurkat cells treated with 0.1 µM STS and 250 ng/mL TNFα were stained with both PI and Annexin-V and analyzed (Figure 16). The percentage of cells that were PI positive was

![Figure 16. 0.1 µM STS and 250 ng/mL TNFα dual treated, stained Jurkat cells indicate cell death.](image)

Jurkat cells were pre-treated (24 h) with a STS (0.1 µM) and TNFα (250 ng/mL) combination, processed for surface staining with both FITC and PI and flow cytometry performed. **Panel A.** Cells were analyzed using an FL1 laser. The black, blue and red peaks represent unstained and untreated Jurkat cells. The blue, green and yellow peaks represent the dual treated Jurkat cells stained with Annexin-V. Fluorescence was detected using an Accuri flow cytometer. Gating was performed using Cflow Plus software. **Panel B.** Cells were analyzed using an FL2 laser. The black, blue and red peaks represent unstained and untreated Jurkat cells. The green, pink and yellow peaks represent the dual treated Jurkat cells stained with propidium iodine. Fluorescence was detected and gating performed as described for Panel A.
0.15% and the percentage of cells that were Annexin-V positive was 23.5%, indicating early stages of apoptosis. Also, the percentage of cells that were both Annexin-V and PI positive was 76.3%, indicating late stages of apoptosis or death.

Jurkat cells treated with 0.2 µM STS and 500 ng/mL TNFα were stained with both PI and FITC and analyzed (Figure 17). The Cflow Plus software determined the percentage of the population that fell within the positive gate. A

![Graphs A and B](#)

**Figure 17.** 0.2 µM STS and 500 ng/mL TNFα dual treated, stained Jurkat cells indicate cell death. Jurkat cells were pre-treated (24 h) with a STS (0.2 µM) and TNFα (500 ng/mL) combination, processed for surface staining with both FITC and PI and flow cytometry performed. **Panel A.** Cells were analyzed using an FL1 laser. The black, blue and red peaks represent unstained and untreated Jurkat cells. The blue, green and yellow peaks represent the dual treated Jurkat cells stained with Annexin-V. Fluorescence was detected using an Accuri flow cytometer. Gating was performed using Cflow Plus software. **Panel B.** Cells were analyzed using an FL2 laser. The black, blue and red peaks represent unstained and untreated Jurkat cells. The green, pink and yellow peaks represent the dual treated Jurkat cells stained with propidium iodine. Fluorescence was detected and gating performed as described for Panel A.
shift in the peak population indicates apoptosis is occurring. The percentage of cells that were PI positive was 0.77% and the percentage of cells that were Annexin-V positive was 20.1%, indicating early stages of apoptosis. Also, the percentage of cells that were both Annexin-V and PI positive was 78.4%, indicating late stages of apoptosis or death.

Overall, all treatments produced statistically significant greater cell death levels than that of the untreated control (Figure 18). These results indicated higher apoptosis levels in Jurkat cells treated with STS alone or a TNFα/STS dual treatment compared to Jurkat cells treated with TNFα alone. Treatment with 250 ng/mL TNFα only induced 17.5% apoptosis while treatment with 500 ng/mL TNFα only induced 14.2% apoptosis. In contrast, treatment with 0.1 uM STS induced 77.4% apoptosis while treatment with 0.2 uM STS induced 77.7% apoptosis. Concurrently, dual treatment with 0.1 uM STS and 250 ng/mL TNFα induced 76.3% apoptosis while dual treatment with 0.2 uM STS and 500 ng/mL TNFα induced 78.4% apoptosis. Treatment with STS alone or a combination of STS and TNFα induced 80% more apoptosis than treatment with TNFα alone. This is indicative of the ability of staurosporine to elicit an apoptotic response in cultured Jurkat cells.
Figure 18. Percent cell death as measured by Annexin-V-FITC and PI staining. Jurkat cells were either untreated (U) or pre-treated (24 h) with STS (0.1 μM or 0.2 μM) and/or TNFα (250 ng/mL or 500 ng/mL), processed for surface staining and flow cytometry was performed. Fluorescence was detected using an Accuri Flow Cytometer. Gating was performed using Cflow Plus software. A one-way ANOVA was performed ($p \leq 0.05$).
The presence of caspase-3 was higher in Jurkat cells treated with STS or a combination of TNFα/STS compared to Jurkat cells treated with TNFα.

To assess the presence of caspase-3 in treated Jurkat cells, flow cytometry was performed. To set initial parameters, an untreated and unstained control population was analyzed. A healthy population of Jurkat cells was gated based on size and granularity. The percentage of cells that were caspase-3 positive was only 0.1%. Using these parameters, Jurkat cells stained with a caspase-3 primary antibody and Zenon Alexa Fluor 488 rabbit labeling reagent were then analyzed.

When treated with 0.1 µM STS, the percentage of Jurkat cells that were caspase-3 positive was 29.9%. When treated with 0.2 µM STS, the percentage of Jurkat cells that were caspase-3 positive was 24.45%. When treated with 250 ng/mL TNFα, the percentage of Jurkat cells that were caspase-3 positive was 18.85%. When treated with 500 ng/mL TNFα, the percentage of Jurkat cells that were caspase-3 positive was 21.15%. When dually treated with 0.1 µM STS and 250 ng/mL TNFα, the percentage of Jurkat cells that were caspase-3 positive was 30.05%. When dually treated with 0.2 µM STS and 500 ng/mL TNFα, the percentage of Jurkat cells that were caspase-3 positive was 36.65%.

Overall, all treatments demonstrated statistically significant greater caspase-3 levels compared to that of the untreated control (Figure 19). Treatment with 250 ng/mL TNFα demonstrated 19% of the cells had caspase-3 while treatment with 500 ng/mL TNFα demonstrated 21% of the cells had
caspase-3. In contrast, treatment with 0.1 uM STS demonstrated 30% of the cells had caspase-3 while treatment with 0.2 uM STS demonstrated only 25% of the cells had caspase-3. Concurrently, dual treatment with 0.1 uM STS and 250 ng/mL TNFα demonstrated 30% of the cells had caspase-3 while dual treatment with 0.2 uM STS and 500 ng/mL TNFα demonstrated 37% of the cells had caspase-3. Treatment with STS alone or a combination of STS and TNFα showed 30% (statistically significant) higher levels of caspase-3 compared to treatment with TNFα alone.

These results coincide with results obtained for the Annexin V-FITC apoptosis assay. Overall, higher levels of apoptosis were associated with higher levels of caspase-3. Treatment with 250 ng/mL TNFα induced 17.5% apoptosis and demonstrated 19% of the cells had caspase-3 while treatment with 500 ng/mL TNFα induced 14.2% apoptosis and demonstrated 21% of the cells had caspase-3. In contrast, treatment with 0.1 uM STS induced 77.4% apoptosis and demonstrated 30% of cells had caspase-3 while treatment with 0.2 uM STS induced 77.7% apoptosis and demonstrated 25% of the cells had caspase-3. Concurrently, dual treatment with 0.1 uM STS and 250 ng/mL TNFα induced 76.3% apoptosis and demonstrated 30% of the cells had caspase-3 while dual treatment with 0.2 uM STS and 500 ng/mL TNFα induced 78.4% apoptosis and demonstrated 37% of the cells had caspase-3.
Jurkat cells were either untreated (U) or pre-treated (24 h) with STS (0.1 μM or 0.2 μM) and/or TNFα (250 ng/mL or 500 ng/mL), processed for intracellular caspase-3 staining and flow cytometry performed. The population was analyzed and fluorescence was detected using an Accuri Flow Cytometer. Gating was performed using Cflow Plus software. A one-way ANOVA was performed ($p \leq 0.05$).

**Figure 19.** Percentage of cells displaying the presence of caspase-3

(* = Different from U; † = Different from TNFα treatments)
The presence of caspase-8 activity was higher in Jurkat cells treated with STS or a combination of TNFα/STS compared to Jurkat cells treated with TNFα.

To assess the presence of caspase-8 in treated Jurkat cells, flow cytometry was performed. To set initial parameters, an untreated and unstained control population was analyzed. A healthy population of Jurkat cells was gated based on size and granularity. The percentage of cells that were caspase-8 positive was only 0.1%. Using these parameters, Jurkat cells stained with a caspase-8 primary antibody and Zenon Alexa Fluor 488 rabbit labeling reagent were analyzed.

When treated with 0.1 µM STS, the percentage of Jurkat cells that were caspase-8 positive was 13.2%. When treated with 0.2 µM STS, the percentage of Jurkat cells that were caspase-8 positive was 9.8%. When treated with 250 ng/mL TNFα, the percentage of Jurkat cells that were caspase-8 positive was 24.15%. When treated with 500 ng/mL TNFα, the percentage of Jurkat cells that were caspase-8 positive was 17.25%. When dually treated with 0.1 µM STS and 250 ng/mL TNFα, the percentage of Jurkat cells that were caspase-8 positive was 15.7%. When dually treated with 0.2 µM STS and 500 ng/mL TNFα, the percentage of Jurkat cells that were caspase-8 positive was 14.65%.

Overall, all treatments demonstrated statistically significant greater levels of caspase-8 compared to that of the untreated control (Figure 20). Treatment with 250 ng/mL TNFα demonstrated 24% of the cells had caspase-8 while
treatment with 500 ng/mL TNFα demonstrated 17% of the cells had caspase-8. In contrast, treatment with 0.1 uM STS demonstrated 13% of the cells had caspase-8 while treatment with 0.2 uM STS demonstrated only 10% of the cells had caspase-8. Concurrently, dual treatment with 0.1 uM STS and 250 ng/mL TNFα demonstrated 16% of the cells had caspase-8 while dual treatment with 0.2 uM STS and 500 ng/mL TNFα demonstrated 15% of the cells had caspase-8. Treatment with STS alone or a combination of STS and TNFα showed 35% (statistically significant) lower levels of caspase-8 compared to treatment with TNFα alone.

These results coincide with results obtained for apoptosis levels. Lower levels of apoptosis were associated with higher levels of caspase-8. Treatment with 250 ng/mL TNFα induced 17.5% apoptosis and demonstrated 24% of the cells had caspase-8 while treatment with 500 ng/mL TNFα induced 14.2% apoptosis and demonstrated 17% of the cells had caspase-8. In contrast, treatment with 0.1 uM STS induced 77.4% apoptosis and demonstrated 13% of cells had caspase-8 while treatment with 0.2 uM STS induced 77.7% apoptosis and demonstrated 10% of the cells had caspase-8. Concurrently, dual treatment with 0.1 uM STS and 250 ng/mL TNFα induced 76.3% apoptosis and demonstrated 16% of the cells had caspase-8 while dual treatment with 0.2 uM STS and 500 ng/mL TNFα induced 78.4% apoptosis and demonstrated 15% of the cells had caspase-8.
Figure 20. Percentage of cells displaying the presence of caspase-8. Jurkat cells were either untreated (U) or pre-treated (24 h) with STS (0.1 μM or 0.2 μM) and/or TNFα (250 ng/mL or 500 ng/mL), processed for intracellular caspase-8 staining and flow cytometry performed. The population was analyzed and fluorescence was detected using an Accuri Flow Cytometer. Gating was performed using Cflow Plus software. A one-way ANOVA was performed (p ≤ 0.05).
Discussion

This research relied on TAL-1 being expressed in cultured Jurkat cells. Intracellular staining and flow cytometry were used to confirm TAL-1 expression. When probed for TAL-1, there was a 94.67% increase in the number of positive cells compared to the unstained control (Figure 4). These results indicate that TAL-1 is being expressed in the cultured Jurkat cells, as was hypothesized. Normal T-cells do not express TAL-1, but expression is observed in malignant T-cells due to a specific translocation (49). Jurkats are an immortalized T-cell line derived from the blood of a leukemia patient. Therefore, TAL-1 expression was expected to be observed.

The results of this study lead to the conclusion that expression of proteins that have a role in resistance to cell death were altered by various treatments. When Jurkat cells were treated with TNFα alone, there was more TAL-1 (Figure 5), NF-κB (Figure 6), TRAF-2 (Figure 7) and IKKγ (Figure 8) protein expression as compared to the untreated control population. These results are similar to those of Adli et al. (52). Their results showed that TNFα treatment increased expression of both IKK and NF-κB. Their results also showed that when IKK expression was silenced, NF-κB expression levels decreased. Also, similar to the results of this study, are those of Manna et al. (53). Their results showed that
TNFα produced a four-fold increase in expression of both TRAF-2 and NF-κB. In the receptor mediated pathway of apoptosis, TRAF-2 leads to activation of the IKK complex via the NF-κB inducing kinase resulting in activation of NF-κB. Their results also showed that when TRAF-2 expression was inhibited, NF-κB expression levels also decreased. This demonstrates the importance of TRAF-2 to the activation of NF-κB. In addition to our findings supporting the work of Adli et al. (52) and Manna et al. (53), our work also demonstrated the important role TAL-1 may play in aiding in resistance to cell death.

When Jurkat cells were treated with STS alone or with a TNFα/STS combination, there was less TAL-1 (Figure 5), TRAF-2 (Figure 7), IKKγ (Figure 8) and inactive p100 NF-κB (Figure 6) protein expression as compared to the untreated control population. However, there was more active p50 NF-κB (Figure 5) protein expression, compared to the control, demonstrating an increase in activation of NF-κB. While active p50 NF-κB expression was higher compared to the control, it was much less than that seen with TNFα treatments. Peet et al. (54) reported that STS, a general kinase inhibitor, was associated with reduced NF-κB protein expression levels via inhibiting kinases, such as Iκβ, involved in the NF-κB pathway. Inhibition of this pathway may be associated with increased apoptosis levels following STS treatments. Our findings continued this work by determining that STS treatments reduced expression of IKK, TRAF-2 and TAL-1.

The results of this study also suggest that IKKγ and TRAF-2 protein expression levels are TAL-1-dependent. When TAL-1 expression increased, IKKγ and TRAF-2 expression also increased. Concurrently, when TAL-1 expression
decreased, IKKγ and TRAF-2 expression also decreased. These results suggest that TAL-1 may control the expression of IKKγ and TRAF-2. This suggests that TAL-1 may associate with a promoter to transcriptionally enhance expression of both IKKγ and TRAF-2. While the relationship between TAL-1, IKK, and NF-κB has been extensively studied (40, 50-52), the relationship between TAL-1 and TRAF-2 is much less known. TRAF-2 is activated by ASK-1 (7) and is an active component in the phosphorylation and activation of the IKK complex (29). TAL-1 may mediate transcriptional control over TRAF-2 increasing expression and allowing for increased activation of the IKK complex.

The results of this research suggest TAL-1 may play a role in the activation of the proteins involved in the NF-κB signaling pathway. NF-κB levels increased regardless of treatment. The results of this study are opposite what Chang et al. (50) reported. Their research showed that increased TAL-1 expression was associated with almost 80% lower p50 NF-κB expression. Concurrently, when TAL-1 was silenced, there was upwards of a 90% increase in p50 NF-κB expression observed. Our results indicate that when TAL-1 expression was higher, NF-κB expression was also higher. However, the results of this study are similar to what O’Neil et al. (51) found. Their results indicated that TAL-1 expression was associated with increased NF-κB and IKK expression. TAL-1, a transcription factor, can associate with the NF-κB promoter (50) and mediate transcriptional activation or repression (40). Our results suggest that TAL-1 expression increases IKK expression and may also further increase NF-κB activation.
Treatment with TNFα alone increases TAL-1, NF-κB, IKKγ, and TRAF-2 protein expression levels, but does not induce significant cell death (18 – 24%) (Figure 18). This suggests that when TAL-1 expression is high, there is resistance to cell death. TAL-1 has been shown to upregulate NF-κB, IKKγ and TRAF-2 (40). When NF-κB, IKKγ and TRAF-2 protein expression levels are high, the cell is primed for a pro-survival response. Upon binding of TNFα to the TNF-R1 and/or TNF-R2 receptor, cell signaling will result in a NF-κB induced pro-survival response. This would result in reduced levels of apoptosis. Concurrently, treatment with STS alone or a TNFα/STS combination decreased TAL-1, IKKγ and TRAF-2 protein expression levels and increased NF-κB protein expression levels, but induced high levels of cell death (76 – 78%) (Figure 18). These results are similar to those shown by Bruno et al. (28). Their results showed that STS treatments induced almost 50% cell death in Jurkat cells. Our results indicated that when TAL-1 expression was low, resistance to cell death may be bypassed. Without TAL-1 transcriptional control, upregulation of NF-κB, IKKγ and TRAF-2 expression levels may not occur. Without TNFα treatment, cell signaling may not be enhanced. At the same time, STS, a general kinase inhibitor, may inhibit the function of the mitogen kinase ASK-1. This could result in a lack of TRAF-2 and IKK complex activation leaving NF-κB sequestered within the cytoplasm unable to elicit a pro-survival response. This could result in the higher levels of apoptosis seen in STS or dual treated extracts.

Apoptosis is a cellular process controlled by numerous caspases (5). This research focused on the receptor mediated pathway of apoptosis which involves
initiator caspase-8 and effector caspase-3. Caspase-3 is the effector caspase responsible for protein degradation and ultimately cell death (17). Caspase-8 is the initiator caspase responsible for activating caspase-3 and thus, the caspase cascade (18). In late stages of apoptosis, caspase-3 will be present while in early stages of apoptosis, caspase-8 will be present. This is because once caspase-8 activates caspase-3, caspase-8 will dissociate and revert back to its inactive form. Then, caspase-3 will act to induce cell death.

Overall, both caspase-3 and -8 levels were higher in treated Jurkat cells compared to that of the untreated control (Figure 19, Figure 20). However, caspase-3 levels were higher and caspase-8 levels were lower in Jurkat cells treated with STS alone or a TNFα/STS dual treatment than in Jurkat cells treated with TNFα alone. Additionally, STS alone and the dual treatments resulted in higher levels of cell death. The results of our study are similar to that reported by Legarda-Addison et al. (12). Their study reported that Jurkat cells lacking a functional IKK complex showed increased caspase levels (both caspase-3 and -8) resulting in enhanced cell death. Our results also indicated that when IKKγ expression decreased following STS treatment, caspase-3 and -8 levels increased and apoptosis levels were high (76%-78%).

Concurrently, caspase-3 levels were lower and caspase-8 levels were higher in Jurkat cells treated with TNFα alone than in Jurkat cells treated with STS alone or a TNFα/STS combination. Additionally, TNFα treatments resulted in decreased levels of cell death. This indicates that caspase-8 activity may somehow be blocked. One potential explanation is through the upregulation of
FLIP by TAL-1 or NF-κB. FLIP might inhibit caspase-8 activity resulting in a lack of caspase-3 activation. These results are similar to those of Travert et al. (55). Their investigation reported that upregulation of FLIP and Bcl-xL resulted in decreased caspase activation and increased NF-κB activation.

In conclusion, the results of this study suggest that TAL-1 alters the expression of proteins involved in the NF-κB signaling pathway (TRAF-2 and IKKγ) and thus, inhibition of cell death. TAL-1 might accomplish this by associating with promoters and causing upregulation of various genes. Treatment with TNFα can then associate with TNF-R1 (4) and influence activation of NF-κB, TRAF-2 and IKKγ (30). Together, enhanced TAL-1 transcription along with TNFα activation may ultimately lead to a more robust NF-κB induced pro-survival response.

The proposed model of TNF-R1 signaling following treatment with TNFα alone is shown in Figure 21. When TNFα binds TNF-R1, receptor dimerization occurs. The adaptor proteins FADD and TRADD are then recruited to associate with the receptor via their DD. Pro-caspase-8 is then recruited to associate with the adaptor proteins via its DED. Normally, bringing pro-caspases within close proximity to one another would result in autoproteolysis. However, it is postulated that the upregulation of FLIP by NF-κB and/or TAL-1 inhibits caspase-8 activity. Essentially, FLIP blocks the activation of caspase-8 and thus, activation of caspase-3 does not occur. Concurrently, the regulatory subunit of the IKK complex, IKKγ/NEMO may also be upregulated by TAL-1. IKKγ/NEMO prevents
RIP, a protein able to activate caspase-8 independent of autoproteolysis, from associating with and activating caspase-8. If higher expression levels of

**Figure 21.** Model of TNF-R1 signaling in Jurkat cells following TNFα treatment.
IKKγ/NEMO are produced, then reduced activation of caspase-8 results. Thus, apoptosis is prevented. This inhibition by FLIP allows a pro-survival response to be sent. TRAF2, which may be upregulated by TAL-1, can associate with the adaptor proteins at the receptor. The kinase, ASK1, will activate TRAF2 which then activates the kinase NIK. This is the first essential step in activating NF-κB and the prosurvival response. Once NIK is activated, it will activate the IKK complex. The IKK complex will then phosphorylate and degrade IκB resulting in the release and translocation of NF-κB to the nucleus. Here, NF-κB can function as a transcription factor to upregulate the expression of anti-apoptotic proteins such as FLIP and XIAPs. Thus, apoptosis is inhibited and the cell survives.

The proposed model of TNF-R1 signaling following treatment with STS alone is shown in Figure 22. With this treatment, there is no presence of TNFα,
so there is no activation of the TNF-R1 receptor. TRAF2, NIK, IKK complex, and NF-κB would not be activated. TAL-1 expression may upregulate the expression of TRAF2 and IKKγ, but there is no activation signal sent from the binding of TNFα to TNF-R1. Without active IKKγ/NEMO, RIP is free to associate with procaspase-8 resulting in activation. NF-κB would remain sequestered within the cytoplasm and therefore not induce transcription of anti-apoptotic factors FLIP.
and XIAPs. Active caspase-8 could then activate caspase-3, resulting in apoptosis. Concurrently, STS will be free to associate with and inhibit the activity of any kinase within the cytoplasm.

The proposed model of TNF-R1 signaling following dual treatment with TNFα and STS is shown in Figure 23. When TNFα binds TNF-R1, receptor dimerization occurs. The adaptor proteins FADD and TRADD are recruited to associate with the receptor via their DD. Pro-caspase-8 is then recruited to associate with the adaptor proteins via its DED. With active NF-κB, FLIP would be upregulated to prevent caspase-8 activity. However, treatment with STS, a general kinase inhibitor, blocks the activation of NF-κB. STS does so by inhibiting the activity of ASK-1, NIK, or the IKK complex. If ASK-1 is inhibited, TRAF2 will not be activated. If NIK is inhibited, regardless of TRAF2 upregulation by TAL-1, the IKK complex will not be activated. Lastly, if the IKK complex is inhibited, not only will RIP be free to associate with pro-caspase-8, but regardless of upregulation of IKKγ by TAL-1, NF-κB will not be activated. NF-κB will remain sequestered within the cytoplasm unable to influence expression of anti-apoptotic factors such as FLIP and XIAP. Therefore, even though the survival signal is initiated by TNFα binding to TNF-R1, activation of NF-κB is blocked due to inhibition of kinase activity by STS. Concurrently, pro-caspase-8 can be activated by autoproteolysis or RIP. Active caspase-8 can then activate caspase-3 resulting in apoptosis.
In summary, TNF-R1 signaling varied following TNFα and/or STS treatment. The TNF-R1 signal following treatment with TNFα alone (Figure 21) is different from the TNF-R1 signal following treatment with STS alone (Figure 22). Furthermore, the TNF-R1 signal following a dual treatment with TNFα and STS
(Figure 23) is again different from the TNF-R1 signal following solitary
treatments. From a clinical standpoint, STS has shown to be too toxic to be used
as a solitary chemotherapeutic drug (24-28). However, if lower and less toxic
concentrations of STS can be used alongside TNFα, there may be a better
response to treatment. TNFα can initiate a signal through the TNF-R1 receptor
and STS could ensure that the signal leads to apoptosis through the inhibition of
ASK-1, NIK, or the IKK complex.

This is significant because TAL-1 is ectopically expressed in 60% of T-ALL
patients (2). Statistically, T-ALL patients respond poorly to traditional
chemotherapy (3). Upregulation of various genes by TAL-1 likely contributes to
increased NF-κB survival signals and thus, decreased apoptosis levels. This
suggests TAL-1 expression is correlated with the poor chemotherapy response.
In the future, research should focus on silencing TAL-1 and repeating these
experiments. To determine if TAL-1 expression truly has an effect on the
expression levels of NF-κB, TRAF-2, and IKKγ along with levels of cell death,
TAL-1 activity needs to be turned off and protein levels assessed again. If results
are similar, TAL-1 may not be exhibiting transcriptional control as demonstrated
by this study. However, if results are opposite to what is reported here, it would
further confirm the importance of TAL-1 in the NF-κB signaling pathway and
apoptosis resistance. It is hoped that the findings of this research will help in the
advancement of more effective, targeted therapies for T-ALL and a better
understanding of how TAL-1 expression contributes to the NF-κB pro-survival
response.
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