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Effect of Anthrax Lethal Toxin on Activation of MAPK Pathway and Autophagy in AML Cell Lines

By

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Effect of Anthrax Lethal Toxin on Activation of MAPK Pathway and Autophagy in AML Cell Lines

Rania Soukarieh

ABSTRACT

Anthrax lethal toxin is an A-B toxin consisting of protective antigen (PrAg), the cell binding and translocation domain, and lethal factor (LF), the catalytic domain, which cleaves all mitogen-activated protein/extracellular regulated kinase kinases (MEKs), leading to the inhibition of the MAPK pathway. Anthrax lethal toxin has been extensively studied for its antitumor effect and showed potency against the majority of acute myeloid leukemia cell lines. In addition to that, previous data have shown that autophagy is activated in most AML cell lines. In this study we investigate the effect of anthrax lethal toxin on the downstream effectors of MEK in the Ras-Raf-MEK-ERK pathway, investigate the effect of MEK inhibition on the downregulation of ERK and try to explore the mechanism underlying autophagy which plays a protective role at later time points, as previously shown. This study aims to explain the difference in susceptibility of different AML cell lines. Flow cytometry analysis of downstream effectors of MEK showed decrease in phosphorylation of ERK and its downstream effectors only at early time points in sensitive cell lines. Whereas this pattern was not observed in resistant cell lines that showed a

constant level of activation up to 120 hours after treatment. Finally, the effect on autophagy is to be more studied by testing for the expression of more proteins involved.

Key Words: AML, Anthrax Lethal Toxin, MAPK Pathway, Autophagy, MEK1/2, ERK1/2, LC3

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LIST OF ABBREVIATIONS

Letx	Anthrax Lethal Toxin
PrAg/LF	Furin-Activated Anthrax Lethal Toxin
PrAgL1/LF	Matrix Metalloprotease-Activated Anthrax Lethal Toxin
PrAgU2/LF	Urokinase-Activated Anthrax Lethal Toxin
MMPs	Matrix Metalloproteinases
uPA	Urokinase Plasminogen Activator
AML	Acute Myeloid Leukemia
MAPK	Mitogen-Activated Protein Kinase
JNK	C-Jun N-Terminal Kinase
TEM8	Tumor Endothelial Marker-8
CMG2	Capillary Morphogenesis Gene 2
RTKs	Receptor Tyrosine Kinases
EGFR	Epidermal Growth Factor Receptor
MEK	Mitogen-Activated Protein Kinase Kinase
ERK	Extracellular Signal-Regulated Kinase
Fp59	Pseudomonas Aeruginosa Exotoxin A
CQ	Chloroquine
PrAg	Protective Antigen
LF	Lethal Factor

EF	Edema Factor
MPO	Myeloperoxidase
CR	Complete Remission
MRD	Measurable Residual Disease
GO	Gemtuzumab Ozogomycin
mTOR	Mammalian Target Of Rapamycin
Atg	Autophagy Related Proteins
PAS	Pre-Autophagosomal Structure
LC3	Microtubule-Associated *Protein* 1A/1B-Light Chain 3

Chapter One

Introduction

1.1 AML

1.1.1 Definiton

Acute myeloid leukemia is a bone marrow disorder, specifically affecting hematopoietic stem cells. It results in the excessive production of malignant clonal myeloid stem cells with an arrest of differentiation (Pelcovits & Niroula, n.d.).

1.1.2 Statistics and Epidemiology:

Until 2017 after being surpassed by CLL, AML had the highest incidence among adults in the United States compared to other subtypes of leukemia namely acute lymphocytic leukemia [ALL], chronic myeloid leukemia [CML], and chronic lymphocytic leukemia [CLL]. In 2019, it was estimated that around 21,000 adults were diagnosed with AML (11,650 males and 9800 females). Of all leukemic deaths, AML accounts for 62% which is the highest percentage among all subtypes (Shallis et al., 2019).

AML principally affects older adults. For 100,000 person years incidence of AML is 20.1 for those aged 65 and above compared to 2.0 for those younger than 65 years old (Shallis et al., 2019).

Population-based studies in a wide variety of countries including US, Canada, Denmark, Australia and Algeria showed that the incidence of AML is 1.2-1.6 times higher in males than in females. These values are also dependent on race and ethnicities (Shallis et al., 2019).

1.1.3 Pathophysiology:

AML patients are presented with signs and symptoms that reflect an overproduction of malignant myeloid cells that are not fully differentiated, accumulating in the bone marrow, the peripheral blood and to a lesser extent in other organs. Leukocytosis, anemia and thrombocytopenia are all

seen presented in patients of AML, in addition to fatigue anorexia and weight loss. Lymph nodes and other organs are not usually affected by size. If left untreated, AML can lead to death as a result of infection or bleeding (De Kouchkovsky & Abdul-Hay, 2016).

1.1.4 Diagnosis:

In the initial examination of a bone marrow sample, based on a 500-cell count with suspected AML, the first step should be to exclude completely the erythroid component, examine the non-erythroid component. If ≥ 20% are blast cells then a diagnosis of AML can be made. Then in order to further diagnose AML, the myeloid origin of these cells should be confirmed. This is done by immunophenotyping where blasts express one and usually more pan-myeloid antigens including, CD_{13}, CD33, and immunohistochemistry to test for myeloperoxidase MPO activity. However, an AML diagnosis can be made regardless of the blast count, depending on the presence of certain unique chromosomal abnormalities such as t(8;21), inv(16) or t(15;17) in the bone marrow (De Kouchkovsky & Abdul-Hay, 2016).

1.1.5 Classification:

According to the French–American–British classification system which was established in 1976, AML is classified, based on differences in cyto-morphologic characteristics of leukemic cells, into 8 variants: (De Kouchkovsky & Abdul-Hay, 2016)

1- M_0: Acute Myeloblastic Leukemia, minimally differentiated (without maturation and with minimal differentiation), accounts for 3% of all AML cases.

2- M_1: Acute Myeloblastic Leukemia without maturation accounts for 15% of total AML cases.

3- M_2: Acute Myeloblastic leukemia with significant maturation, accounts for 30% of total AML cases including the M2 variant with eosinophilia.
4- M_3: Acute Promyelocytic leukemia, accounts for 10% of total AML cases including both the hypo-granular and the hyper-granular variants.
5- M4: Acute Myelomonocytic Leukemia, accounts for 25% of total AML cases inc;uding the M_4 variant with eosinophilia
6- M_5: Acute Monocytic leukemia, accounts for 10% of total AML cases including both subtypes M_5a and M_5b
7- M_6: Acute erythroleukemia, accounts for 5% or less of total AML cases.
8- M_7: Acute Megakaryoblastic leukemia, accounts for 3% or less of AML cases.

Then later in 2001, the World Health Organization WHO suggested a classification that is not only based on morphology and cyto-chemical characteristics but also incorporates genetic information, the immunophenotype and the clinical presentation of the patient. This classification was made based on advances in diagnosis and therapy. Now, six major entities of AML are recognized: (1) AML with recurrent genetic abnormalities, (2) AML with myelodysplasia-related changes, (3) Therapy-related myeloid neoplasms, (4) AML Not Otherwise Specified, (5) Myeloid sarcoma, and (6) Myeloid proliferations related to Down syndrome. These major entities are further classified into more specific subtypes based on chromosomal translocations. (De Kouchkovsky & Abdul-Hay, 2016)

Another criterion to classify AML is one that is based on prognostic factors. Based on their cytogenetic profile, 3 prognostic risk groups of AML are identified: favorable, intermediate, and adverse. For example, AML cases presented with chromosomal rearrangements such as t(8;21), t(15;17) or inv(16) all assumed to have a favorable prognosis having a 3 years overall survival in

66% of patients younger than 60 years old and 33% in those older than 60 years old. Whereas patients having a complex karyotype, one having three or more chromosomal rearrangements, monosomy 5 or 7, t(6;9) or inv(3) all demonstrated a higher possibility of treatment failure and death. (De Kouchkovsky & Abdul-Hay, 2016)

This classification was essential for a better management of AML. Prognostic factors help physicians decide what type of therapy this specific patient needs by assessing their risk of developing resistance toward a certain treatment protocol and assessing the chance of treatment-related mortality.

1.1.6 Treatment:

Treatment protocols of AML basically consist of an initial induction therapy that aims for complete remission (CR) with no measurable residual disease (MRD) in a best case scenario (Pelcovits & Niroula, n.d.). Regardless of the type of induction therapy, improved survival was reported in patients who succeeded in achieving CR (Othus et al., 2016). The type of initial induction therapy chosen is actually dependent on several factors including patient functional status, as in performance and comorbidities, prognostic risk groups and the molecular profile of leukemic cells that eventually assess the biological status of the disease. The 2 most commonly used induction therapies in AML cases are cytotoxic chemotherapy and hypomethylating agents, both with or without targeted therapies (Pelcovits & Niroula, n.d.).

Induction chemotherapy known as 7+3 has been quite the same over the past 50 years. It consists of Cytarabine infusion over the course of 7 days while administering Daunorubicin, an anthracyclin, daily during the first 3 days. Patients with favorable risk disease show full disease response, in 80% of cases, upon the 7+3 therapy, compared to 50-60% of those with intermediate adverse risk disease (Fernandez et al., 2009). However, the standard 7+3 chemotherapy did not

show satisfactory outcomes in adverse risk disease where only 40% of cases showed complete remission and the median overall survival ranges between 12 and 18 months (Pelcovits & Niroula, n.d.).

However, due to their poor selectivity for tumor cells versus normal ones, the success of mainstream therapeutic approaches including surgery, chemotherapy and irradiation remain unfortunately limited. This has resulted in either systematic toxicity, ineffective drug concentrations in tumor cells, and emergence of drug-resistant tumor cells (Xu & McLeod, 2001). Targeted cancer therapy targets specific genes or proteins in tumors cells that are supposedly promote tumor growth. Using monoclonal antibodies or oral small drugs, targeted cancer therapeutics aim to inhibit the proliferation of tumor cells, enhance cell cycle regulation, promote cell death by apoptosis or induce autophagy (Gerber, 2008; Padma, 2015).

For example, the addition of targeted drugs to the traditional induction chemotherapy which is commonly known as 7+3 improved the treatment outcomes in both the favorable and intermediate risk groups. Examples include Gemtuzumab ozogomycin (GO) and Midostaurin (Pelcovits & Niroula, n.d.).

GO is a monoclonal antibody that recognizes an antigen on the surface of myeloid leukemic cells, CD33. Decreased risk of relapse and improved overall survival were reported upon the inclusion of GO to the conventional protocol of chemotherapy in favorable and intermediate risk groups with better results in the favorable risk group compared to the intermediate one (Castaigne et al., 2012).

On the other hand, Midostaurin is an example of oral drugs that are inhibitors of tyrosine kinases. It is active primarily in patients having a FLT3 mutations. Upon addition of Midostaurin to the standard 7+3 chemotherapy, survival rates have increased from 25 to 74 months in AML patients

with FLT3 mutations. This highlights the importance of early cytogenetic and molecular profiling of leukemic cells in each patient as these drugs can be added to the first day of chemotherapy (Stone et al., 2017).

1.2 MAPK Pathway

Different intracellular signaling pathways play different roles in regulating the dynamics of cancer such as proliferation, survival, differentiation, and motility of cancer cells. Among these is the RAS/RAF/MAP kinase-ERK kinase (MEK)/extracellular-signal-regulated kinase (ERK) (MAPK) pathway (De Luca et al., 2012). Activation of MAPK signaling cascade requires a ligand to bind to the tyrosine kinase receptor (RTKs), specifically to its extracellular domain. Ligands can be hormones, growth factors or chemokines. Once bound to its ligand the RTK dimerizes and stimulates RAS activation (Shaw & Cantley, 2006). C-terminal tyrosine residues are now phosphorylated and thus constitute docking sites for proteins that contain Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains. The adaptor protein growth factor receptor-bound protein 2 (GRB2) is an example (Shaw & Cantley, 2006). Further effectors, such as the guanine-nucleotide exchange factor son of sevenless (SOS) are then recruited (Katz et al., 2007). At the plasma membrane, where RAS proteins are localized and SOS is recruited, a nucleotide exchange of GDP to GTP on RAS takes place. This would convert RAS from a GDP-bound inactive form to a GTP-bound active one (Downward, 2003). Then, in this active conformation, RAS proteins recruit RAF protein kinases, namely CRAF, BRAF and ARAF, to the cell membrane, where the catalytic kinase activity of RAF is completed by number of membrane-associated events (Downward, 2003). RAF, then, by phosphorylation, switch on the MAPK kinases/extracellular-signal-regulated kinases (MEK1 and MEK2). In turn, MEKs phosphorylate tyrosine and threonine residues of MAPKs, ERK1 and ERK2, thus activating them (Downward, 2003; Katz et al., 2007).

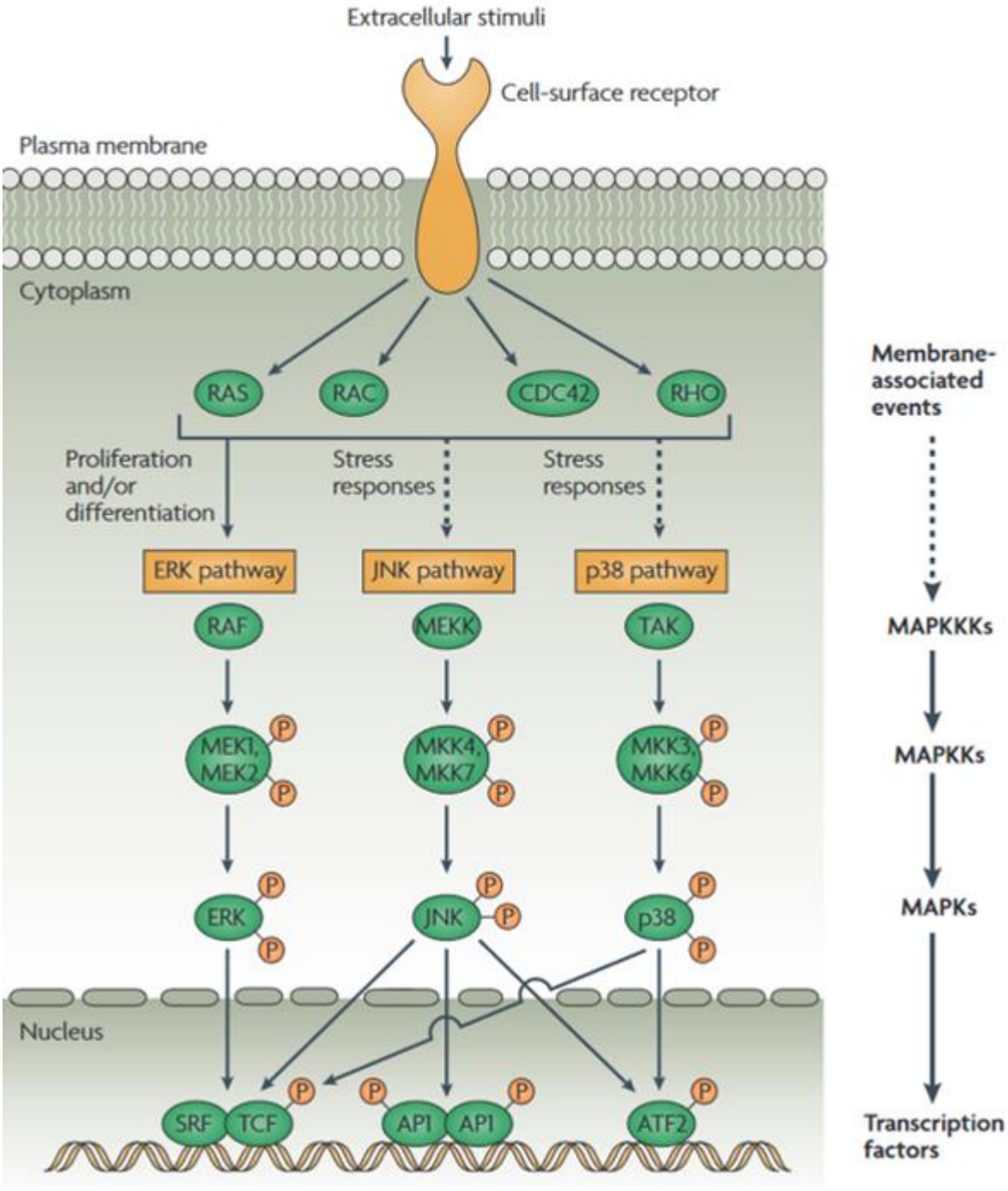


Figure 1: Schematic diagram of the three mammalian mitogen-activated protein kinase (MAPK) signalling pathways adapted from (Y. Liu et al., 2007)

1.2.1 MAPK Pathway Mutations:

Several biological events cause the MAPK pathway to be constitutively active. For example, increased activation of RTKs, EGFR for instant, because of mutations, gene amplification or increased signaling can result in the constitutive activation of the MAPK pathway. In addition to that, each and every member of this cascade, namely RAS, RAF and MEK genes, is prone to mutations that will also eventually cause the constitutive activation of this pathway (Katz et al., 2007).

RAS family gene mutations have been previously reported and detected in all members of this family: HRAS, KRAS and NRAS. The vast majority of these mutations were detected in the 12^{th}, 13^{th}, or the 61^{st} codons of the RAS gene. These have led to single amino acid substitutions that impaired GTP hydrolysis by GTPase (Normanno et al., 2009). Approximately 30% of human cancers were found to have RAS mutations where KRAS mutations being that most abundant, accounting for 85% of total RAS mutations. KRAS mutations are most abundantly detected in pancreatic cancers where 69% of cases had KRAS mutations. NRAS mutations, being the second most common of the RAS mutations, are found in 19% of melanoma cases (De Luca et al., 2012). On the other hand, BRAF mutations were also detected in wide range of human carcinomas, mainly in melanomas, where 60% of cases are presented with BRAF mutations. Of these mutations, V600E is the most abundant and accounts for 90% of all BRAF mutations detected in human cancers. This happens when glutamic acid replaces valine at the residue 600, thus causing the constitutive activation this pathway (Davies et al., 2002).

MEK and ERK mutations, unlike RAS and RAF mutations, are far less common in cancer genomes. Evidence suggest that they are cancer drivers because they never co-occur with Ras or Raf mutations (Brown et al., 2014).

In the case of AML specifically, no significant MAP kinase pathway mutations are reported to this

date, even though AML cells are dependent on this specific pathway for survival. However, In a previous study, it was shown that 2 out of 10 AML samples that exhibit constitutive activation of the MAP kinase pathway, had N-*ras* mutation at the codon number 12 where Asp replaced Gly (Towatari et al., 1997). Other mutations reported in some AML cases were FLT3 mutations. FLT3 or FMS-like tyrosine kinase 3 is an RTK that is activated following the binding of its ligand FL. It is expressed on both normal hematopoietic stem cells and acute leukemia cells (Kiyoi et al., 2020). 20% of AML cases are reported to have the FLT3-ITD mutation which occurs in the domain that codes for juxtamembrane JM domain (Nakao et al., 1996). Later on, a study showed that only 10% of AML cases were proven to contain the *FLT3*-TKD mutation that results from several deletion, insertions and point mutations around D835 residue in the domain coding for tyrosine kinase (Kihara et al., 2014). In another study, it was shown that AML cells with mutant FLT3 showed a constitutively active MAP kinase pathway (Hayakawa et al., 2000).

1.3 Anthrax Lethal Toxin

To promote virulence, protein toxins are frequently produced by infectious bacteria, making it easier to evade the immune system of the host. These toxins contribute to bacterial dissemination and to overall disease pathology. A-B toxins are in general proteases that once recognize a target cell and enter it; they manage to disable its function. They do so by targeting host substrates, usually intracellular proteins, under the action of their enzymatic subunits. These toxins act on a very well-defined set of substrates making their mode of action selective and specific.

Bacillus anthracis, which is a gram-positive rod-shaped bacterium, produces anthrax lethal toxin (Letx). LeTx is an A-B toxin formed of 3 general moieties: the cell binding moiety which is the protective antigen (PA, 83 kDa), the enzymatic or the catalytic moiety which is the lethal factor (LF, 90 kDa), and finally the edema factor (EF, 89 kDa). Separately, these three polypeptides are

non-toxic.

1.3.1 Structure:

Protective antigen PrAg is a flat long protein that has a lot of beta-sheets in structure. This structure was extensively studied by X-ray diffraction and based on this, four different domains corresponding to different functional regions were identified and defined (Liu, Schubert, et al., 2003).

Domain 1 which comprises residues 1 to 258 has 2 calcium ions tightly bound together in addition to a huge flexible loop involving residues 162 to 175. This loop has the sequence RKKR that is recognized and cleaved by furin or furin like proteases when PrAg binds to its specific receptors on the cell surface. This cleavage results in 2 fragments: the 20 kDa fragment or the amino terminal fragment that is left in the extracellular space, and the 63 kDa fragment that remains attached to the receptor and then associates with other 63 kDa fragments to form heptamers of octamers. This cleavage step is specifically important due to the fact that LF and EF exclusively bind to PrAg heptamers/ octamers and never to monomers, while the presence of the amino NH2 terminal hinders PrAg oligomerization, and as such it has to be removed. 3 and 4 LF or EF molecules can bind to one PrAg heptamer and octamer respectively. Not only is the step of oligomerization important for the binding of LF and EF, but also for the internalization of the complex into the cytoplasm (Liu, Schubert, et al., 2003).

Domain 2 has long beta-sheets and covers residues 259 to 487. This domain is found to be involved in membrane insertion and channel formation and similar to domain 1, it has a large flexible loop over the residues 303-319 (Liu, Schubert, et al., 2003).

Domain 3 which consists of residues 488 to 595 was found to be implicated in the formation of PrAg oligomers (Liu, Schubert, et al., 2003).

Finally, domain 4 which constitutes of residues 596 till 735 is found to be involved in receptor binding and is loosely bound to the other 3 domains (Liu, Schubert, et al., 2003).

On the other hand, LF is a zinc dependent metalloprotease. Once inside the host cell, it cleaves specifically a number of proteins including six MKKs the mitogen-activated protein kinase kinases (MEK1/2 and MKK3/4/6/7) in their amino terminal region. The sites where the interaction of MKKs and their MAPK substrates take place are specifically targeted by LF and as such, the effect of LF terminates signaling through ERK1/2, p38, and JNK pathways which are the 3 major MAPK pathways (Goldberg et al., 2017).

1.3.2 Mode of Action:

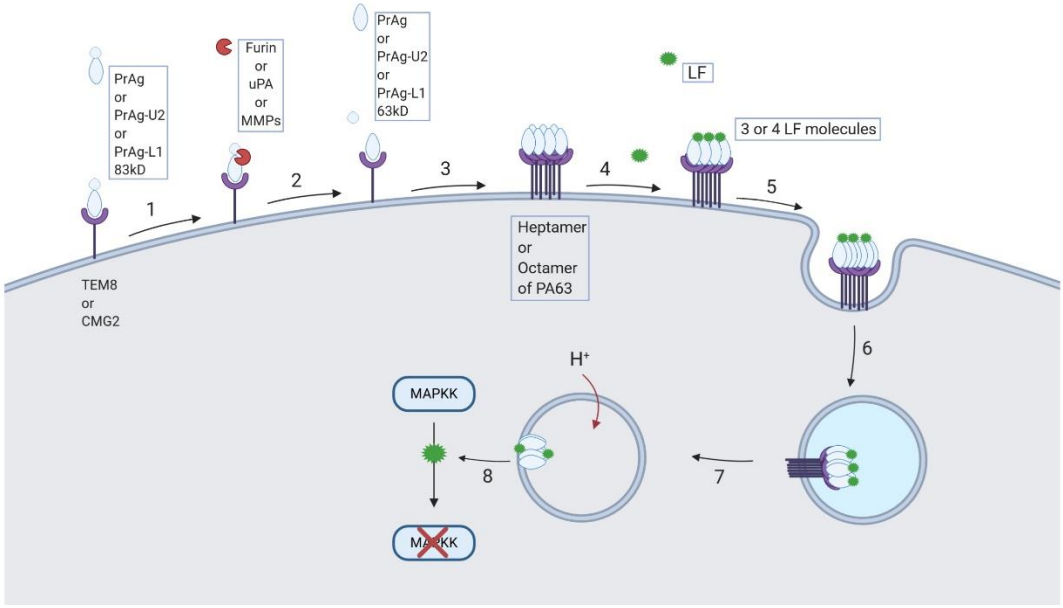


Figure 2: Mechanism of action of Anthrax Lethal Toxin

First, the 83 kDa PrAg fragment binds to the receptors (TEM8 or CMG2) on the surface of the host cell. Then, at the RKKR sequence, PrAg is recognized and cleaved by furin or furin-like receptors releasing the 20 kDa fragment into the medium. Heptamers or octamers are assembled by self-association of 63 kDa fragments. 3 or 4 LF units bind to the heptamers or the octamers respectively and then by receptor-mediated endocytosis, the whole complex is internalized. Due

to a drop in pH inside the endosome, which contains the complex, a conformational change occurs and a channel is formed in the membrane on the endosome. This channel functions to transport the LF molecules into the cytosol. Now LF is ready to cleave its substrates in the cytosol. (Liu, Schubert, et al., 2003)

1.3.3 Derivatives:

Even though this study was carried using only wild type anthrax lethal toxin, it is quite important to discuss how it was engineered and modified to increase its selectivity. Protease systems such as matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA) are over produced by many cell lines and tumor tissues. These systems are greatly related to cancer cell growth, tumor invasion and metastasis. Taking advantage of this characteristic while taking into consideration that the protective antigen PrAg has to be cleaved at the target cell surface, the latter was extensively studied and re-engineered to produce tumor cell-selective cytotoxins. This was done by altering the furin cleavage site in the PrAg and replacing it by sequences recognized and cleaved by these proteases, MMPs or uPA. These mutated versions, detailed below, did not target non-tumorigenic normal cells that do not overexpress any of the mentioned protease systems when co-cultured with tumor cells expressing them (Liu, Aaronson, et al., 2003; Liu et al., 2000).

MMPs are zinc-dependent endopeptidases found to be implicated normal and pathological tissue remodeling including normal tissue repair and ECM impairment (Birkedal-Hansen, 1995).

A wide variety of enzymes, including stomelysins, collagenases, membrane-type metalloproteinases and gelatinases, constitute the family of MMPs (Birkedal-Hansen, 1995).

As previously mentioned, various tumor cell lines and tissues highly express MMPs that are involved in metastasis and tumor invasion (Stetler-Stevenson et al., 1993). The most MMPs found to be implicated in human cancer metastasis are MMP-2 (gelatinase A), MMP-9 (gelatinase B),

and membrane-type 1 MMP (MT1-MMP) (Jones et al., 1999).

In an attempt to increase the selectivity of anthrax lethal toxin, as a potential anti-cancer drug, 2 mutated anthrax PA proteins were constructed. PA-L1 was generated by replacing the furin cleavage site RKKR by GPLGMLSQ that is recognized and cleaved by MMP-2 (gelatinase A). Whereas, PA-L2 was generated by replacing the furin cleavage site RKKR by GPLGLWAQ that is recognized and cleaved by MMP-9 (gelatinase B) (Liu et al., 2000). These mutants, when combined with FP59, were able to selectively kill tumor cell lines that overexpressed MMPs including fibrosarcoma HT1080 cells, breast cancer MDA-MB-231 cells, and melanoma A2058 cells (Liu et al., 2000). As such, this engineered version of PA was less toxic to mice models than the wild type due to the fact that normal cells express MMPs in a limited manner (Liu et al., 2000). Add to this, the MMP-activated version of PA showed anti-tumor activity to other tumor types and not only those with the BRAF mutation such as lung and colon carcinomas irrespective of whether they do or do not have BRAF mutations (Liu et al., 2000).

MMP-activated LeTx, via *in vivo* efficacy studies, showed that endothelial cells migrated less into the tumor parenchyma and thus inhibited tumor xenografts growth. It also reduced endothelial proangiogenic MMP expression thus decreasing the endothelial invasive capacity (Liu et al., 2000).

1.3.4 Previous Work on Anthrax Lethal Toxin:

Several previous studies on anthrax lethal toxin showed the great potential it has as a cancer targeted therapeutic and a possible alternative for standard therapy protocols. For example, 11 out of 18 melanoma cell lines were sensitive to treatment with anthrax lethal toxin compared to only 5 out of 15 normal cell lines. This suggests that the cytotoxicity of PrAgLF is selective to melanoma cell lines and suggests the potential use of PrAgLF as an attractive alternative for

melanoma therapy (Abi-Habib et al., 2005).

Furthermore, partial and complete tumor regressions were reported in athymic nude mice with s.c. xenograft melanoma upon *in vivo* systematic treatment with LeTx. This was also with minor toxicity to mice, no histological evidence of tissue damage. And as such, this study showed for the first time, that *in vivo* systematic treatment with LeTx could be a promising antimelanoma agent awaiting for further preclinical development (Abi-Habib et al., 2006).

Renal cell carcinoma RCC is another tumor type that was tested against LeTx. Cell proliferation decreased by 20% upon 72 h treatment with LeTx with no effects on apoptosis or cell cycle distribution. A dramatic inhibition of anchorage-independent growth of renal cell carcinoma cells was also observed under the effect of anthrax lethal toxin. In addition to that, LeTx treatment caused a decrease in tumor neovascularization and increase in necrosis (Huang et al., 2008).

In addition to that, a major decrease in cell motility and invasion across a collagen matrix was observed upon LeTx treatment in astrocytoma cells (Al-Dimassi et al., 2016).

Another study on the effect of LeTx on breast cancer cells reported a major decrease in motility and invasion and an increase in adhesion all mediated by LeTx treatment (El-Chami et al., 2021).

1.4 Autophagy

Autophagy is a catabolic process, occurring intracellularly, that involves the degradation of cytoplasmic components such as pathogens, malfunctioning organelles, aggregated proteins, or macromolecules. Products of the autophagy process are provided back to the cell in the form of ATP, nucleotides, amino acids or sugars (Li et al., 2020). While autophagy can be classified into various selective types with different target cargo, as the substrates to be degraded, the term autophagy is often referred to as macroautophagy.

1.4.1 Mechanism of Autophagy:

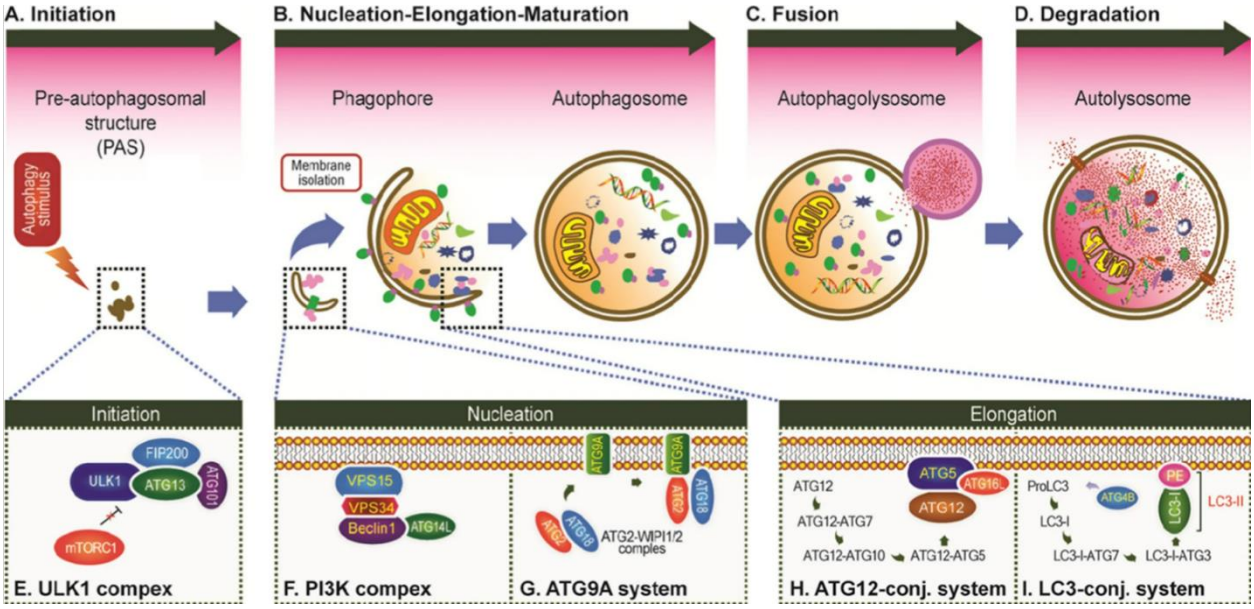


Figure 3: Schematic overview of autophagy adapted from (Li et al., 2020)

Autophagy is a series of five sequential and closely related steps. Starting with induction of autophagy, passing by the formation and the assembly of the autophagosome and its fusion with the lysosome until reaching the step of cargo degradation, the whole process of autophagy is orchestrated and organized by a group of proteins referred to as autophagy related proteins.

Several stimuli, both intracellular and extracellular, may trigger the induction of autophagy. Examples include nutrient starvation, oxidative stress and TOR inhibitors such as Rapamycin. In all mentioned cases, high levels of autophagy are induced in order to either compensate the depletion of nutrients, or degrade protein aggregates or recycle malfunctioning organelles (Mazure & Pouysségur, 2010; Mizushima, 2007; Ureshino et al., 2014).

Normally, and under optimal growth conditions, mTORC1 kinase in its active form inhibits the induction of autophagy by directly phosphorylating ATG13 and therefor preventing the binding of ULK1 (Atg1 in yeast) and FIP200 and thus inhibiting the formation of ULK1- ATG13 – FIP200 – ATG101 complex that is necessary for autophagy initiation (Kawamata et al., 2008).

However, due to stressful events that render mTORC1 inactive, ATG13 is dephosphorylated and thus ATG13 is again open for the interaction with ULK1 and FIP200 allowing for the formation of the ULK1- ATG13 – FIP200 – ATG101 complex, or the autophagy initiation complex, at the pre-autophagosomal structure PAS. Only now, the rest of autophagy related proteins are recruited in a hierarchical manner to the PAS. These proteins form complexes and functional units necessary for the following steps. Note that the PAS is a cytoplasmic site found to be necessary for vacuole targeting and the formation of the autophagosome (Yamamoto et al., 2016).

At this stage, and following the initiation of the autophagy process, four additional functional units are recruited to the PAS aiming to form a mature autophagosome. These complexes, namely PI3K complex, ATG9A system, ATG12-conjugation system, and LC3-conjugation system are involved in a series of steps including (1) nucleation of the autophagy related proteins ATGs, (2) elongation of the phagophore and (3) maturation of autophagosome (Tooze & Yoshimori, 2010).

First, an isolation membrane or a phagophore is formed upon the gathering of ATGs on the PAS. The nucleation step is initiated upon the formation of the PAS scaffold complex that consists of ULK1 associated with Atg13, FIP200/Atg17, Atg29, and Atg31 (Cheong et al., 2008, p. 1).

Second, ATG14L is recruited to the nucleation site where it interacts and binds to ATG13 and thereafter, the PI3K complex, including Beclin1, VPS34, VPS15, and ATG14L, gathers at the PAS and is also involved in the phagophore formation (Cheong et al., 2008, p. 1).

Then, the ATG9A/ATG2-WIPI1/2 trafficking system that contains ATG9A positive membrane vesicles, ATG2 and WIPI1/2 interacts with FIP200. At the PAS, and in the aim of forming the isolation membrane, ATG9A-containing vesicles fuse and ATGs coordinate so that the membrane elongates and proceeds to engulf the concerned organelles at the specified cytoplasmic site (Cheong et al., 2008, p. 1).

Finally, a mature autophagosome having a double membrane is formed upon the intervention of two ubiquitin-like ATG conjugation pathways, (1) The ATG12-conjugation system that includes ATG12, ATG7, ATG10, ATG5, and ATG16L and (2) LC3-conjugation system which includes ProLC3, ATG4, LC3-I, ATG7, ATG3, and LC3-II (LC3-I/PE) (Suzuki et al., 2013)

The next step is the formation of the autophagolysosome which, as the name indicates, is a fusion of the mature autophagosome and the lysosome. While autophagosomes can be produced anywhere in the cytoplasm, the lysosomes are generally present in the perinuclear area. Due to this fact, once produced, the mature autophagosomes need to be transported to the perinuclear area where they can now dock and fuse to lysosomes and thus form autophagolysosomes (Wijdeven et al., 2016).

Finally, engulfed macromolecules, proteins, organelles and pathogens are degraded under the action of enzymes in the lysosomes such as lysosomal hydrolases. These are broken down into amino acids, nucleic acids and sugars, that through permeases and reverse transported located in the lysosomal membrane, manage to go back to the cytosol to be reutilized by the cell (Li et al., 2020).

1.4.2 Autophagy in Cancer:

By degrading malfunctioning organelles and defective proteins, autophagy serves a list of physiological functions such as maintaining an adaptive metabolic response to stress and starvation, regulating embryonic development and tissue differentiation, sustaining homeostasis, controlling pathogen replication and assisting the immune system in infection and inflammations, and other functions including, but not limited to antiaging and stem cell maintenance (Levine & Kroemer, 2008).

However, what recent evidence has shown is that the role of autophagy is quite context dependent.

This means that in tumor cells specifically, this process can suppress or promote tumor growth or act neutrally depending on a variety of conditions including the stage of cancer development, is type and genetic profilen (Mathew et al., 2007).

1.4.2.1 Autophagy as a tumor suppressor

It was previously reported that certain cancers are associated with down-regulation of genes essential for autophagy and that due to the under expression of autophagy related genes, the chance of developing tumors is elevated. One example is Beclin1, an autophagy related gene. Cases of human hepatocellular carcinoma, breast, ovarian and prostate cancers were reported to have a mono-allelic deletion of the Beclin1 gene (Aita et al., 1999). In another study, 44 patients suffering from hepatocellular carcinoma had decreased expression of Beclin1 gene (Ding et al., 2008). In addition to that, Beclin1+/− mouse models showed a higher tendency to develop malignancies (Yue et al., 2003). Other examples include other ATGs such as ATG5, ATG2B, ATG12 and ATG9B that were found to have frameshift mutations with mononucleotide repeats in gastric and colorectal cancers. This suggests that defective autophagy contributes to tumorigenesis while intact autophagy contributes to tumor suppression (Kang et al., 2009). On the other hand, studies have reported high levels of p62/SQSTM1 in several tumor types such as gastrointestinal, hepatocellular, breast, prostate and lung cancers. p62 (Sequestosome 1 SQSTM1 in humans) is an adaptor protein that is degraded by autophagy upon interaction with LC3. When autophagy is defective, p62 accumulates and this correlate with the progression of cancer. Thus autophagy might act as a tumor suppressor by inhibiting the accumulation of p62 (Bjørkøy et al., 2005).

1.4.2.2 Autophagy as a tumor promoter:

This happens mostly in late stages of cancer progression. First, increased cancer cell survival as a result of autophagy, was reported in many previous studies (Barnard et al., 2016). Apart from that,

autophagy plays a role in promoting metastasis that is correlated with poor prognosis and advanced stages of cancer (Maishman et al., 2017). Steps of malignant cells disseminating into the circulation, colonizing in other organs and then entering a state of dormancy are all promoted by the process of autophagy. Autophagy is upregulated in response to hypoxia, metabolic stress and detachment from the ECM (Fung et al., 2008). This was concluded after analyzing the change in the levels of LC3 which is an autophagy marker, where increased autophagy was observed in breast cancer and melanoma metastases (Lazova et al., 2012, p. 3).

1.5 Previous Work done on Anthrax Lethal Toxin on AML:

The effect of wild type anthrax lethal toxin and its derivatives has been previously tested on AML cell lines. A study showed that approximately 64% AML cell lines showed sensitivity to LeTx and this cytotoxicity was similar to that induced by U0126, a MEK1/2 inhibitor, showing that this cytotoxic response mediated by anthrax lethal toxin is due to inhibition of MAPK pathway, the MEK1/2-ERK1/2 branch specifically. Phospho-ERK1/2 was exclusively detected in cell lines sensitive to LeTx as reported by flow cytometry analysis of MAPK pathway activation (Kassab et al., 2013).

Another study aimed to test the effect of an anthrax lethal toxin derivative, urokinase-activated anthrax lethal toxin PrAgU2/LF, on AML cell lines and primary blasts. It showed that 5 out of 9 AML cells lines were sensitive to this derivative, with nonapoptotic cytotoxicity. Flow cytometry analysis showed (1) higher degree of MEK1/2 phosphorylation in cells lines that PrAgU2/LF was cytotoxic to and (2) expression of uPAR, indicating that this cytotoxicity was linked to both activation of the MAPK pathway and urokinase activity. In addition to that, 4 out 5 AML patient blasts had a cytotoxic response upon treatment with PrAgU2/LF. Similar to AML cell lines tested before, primary blasts sensitivity was dependent on the expression of uPAR and activation of

MAPK pathway. This was not the case for bone marrow and peripheral blood cells that did not express uPAR and thus managed to survive treatment with PrAgU2/LF. As such, and because this derivative was selective and cytotoxic against tumor cells only, it constitutes a potential therapeutic for AML (Bekdash et al., 2015).

1.6 Aim Of the Study:

Looking back at these previous studies, it's important to emphasize the fact that we have sensitive and resistant AML cell lines. However, it is still not clear why this is the case neither what is their mechanism of sensitivity. This study aims to characterize the mechanism of cell death in sensitive cell lines and hence the mechanism of resistance in the resistant ones. We attempt to investigate the effect of anthrax lethal toxin on MEK1/2 and ERK1/2 and study the activation of MAPK pathway through studying the levels of phosphorylation of MEK1/2, ERK1/2 and the downstream effectors of ERK such as p90RSK, while comparing the pattern of activation in both sensitive and resistant cell lines. We also attempt to complete the already started work on the effect of anthrax lethal toxin on the activation of autophagy by studying the expression of the autophagy market LC3.

Chapter Two

Materials and Methods

2.1 Expression and purification of PrAg/LF

Wild type PrAg and LF were expressed and purifiedin the laboratory of Stephen H. Leppla at the National Institute of Allergies and Infectious Diseases (NIAID) of the National Institutes of Health (NIH) in Bethesda, MD,as described previously(Ramirez DM, 2002)(Liu S, 2001).

2.2 Cell Lines

Human AML cell lines Mono-Mac-6, U-937, HL-60 and ML-2 were grown in RPMI 1640 (Lonza, Basel, Switzerland or Sigma-Aldrich) or culture media supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich) and 100U/ml penicillin/sterptomycin (Biowest). Cell lines were incubated at 37°C, 5% CO2.

2.3 Antibodies and Reagents Used

The following primary antibodies were used in this study: rabbit anti-MEK (#8727S), rabbit anti-phospho-MEK (#2338S), rabbit anti ERK (#4695S), rabbit anti-phospho-ERK(#4370S), rabbit anti phosph-MSK1 (#9595S), rabbit anti p-p90RSK (#12032S), all purchased from Cell Signaling Technology (USA), and rabbit anti LC3B (ab51520) purchased from Abcam (USA). Anti-rabbit IgG HRP Conjugate secondary antibodies (W401B) were obtained from Promega (Promega, Madison, WI, USA). Rabbit mAb AgG Isotype Control (#3900S), obtained from Cell Signaling Technology and goat anti-rabbit IgG-FITC (sc-2012), obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA), were also used in this study.

2.4 Western Blots

In brief, cells were treated with PA (10^{-8} M) and LF (10^{-9} M) and harvested at 5 time points, 24 hrs, 48 hrs, 72 hours 96 hrs and 120 hrs. Cells were then lysed in 1x RIPA buffer (50 mM Tris-

HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with protease inhibitors on ice. Protein concentrations of lysates were measured using DC protein assay (500–0112; Bio-Rad Laboratories), and blots were performed using equal amounts of protein (20–40 µg). SDS-PAGE was carried out under standard conditions and proteins were blotted onto a PVDF membrane. The membranes were then blocked with 5% BSA (Sigma Life science #A2153) for 1 hour and then incubated overnight at 4°C or 2 hours at room temperature with gentle shaking with primary antibodies against MEK (1:1000), ERK (1:1000), p-MSK1 (1:1000), and LC3 (1:3000). After incubation with the primary antibody, the membranes were washed and incubated with Anti-rabbit IgG HRP Conjugate secondary antibodies (Promega, W401B) at a dilution of 1:2500 for 1 hour at room temperature. The membranes were then washed, and the bands are visualized by treating the membranes with western blotting chemiluminescent reagent ECL (GE Healthcare). The levels of protein expression were compared by densitometry using the ImageJ software.

2.5 Intracellular Staining and Flow Cytometry:

Cells were plated in flat-bottom 6-well plates (0.75 x 106 cells/well) (Corning Inc. Corning, NY). Conditions included control and PrAg/LF. LF and PA were added when needed to yield concentrations of 10-9 M and 10-8 M respectively. Plates were incubated at 37oC/5% CO2 harvested at 5 time points, 24 hrs, 48 hrs, 72 hours 96 hrs and 120 hrs. Briefly, cells were collected and incubated in dark in antibody binding buffer consisting of 0.05% Triton-X 100 along with rabbit anti pMEK (1:100), rabbit anti pERK (1:800) and rabbit anti p-p90RSK (1:400) for 1 h at 37 °C, followed by a 30-min incubation with a 1:100 dilution of a goat anti-rabbit IgG-FITC antibodies. Isotypic control consisted of cells incubated with Rabbit IgG (Cell signaling Technologies) and goat anti-rabbit IgG-FITC antibodies. Results were read on a C6 flow cytometer

(BD Accuri, Ann Arbor, MI).

Chapter Three

Results

3.1 Effect of PrAg/LF on inhibition of MEK1/2 and ERK1/2:

We examined the effect of anthrax lethal toxin on the expression of MEK1/2 and ERK1/2 in AML cell lines in control untreated cells and following a 24, 48, 72, 96 hours incubation with PrAg/LF using western blots.A representative panel of 3 AML cell lines, namely HL60, U937 and ML2, was tested and the expression of MEK1/2 and ERK1/2 was observed.

In all 3 cell lines, the expression of MEK1/2 decreased dramatically upon treating with anthrax lethal toxin (Figure 4). However, this was not the case for ERK1/2 where all these cell lines did not actually show a decrease in ERK1/2 expression (Figure 5). Then, we examined the activation level of the MAPK pathway in AML cell lines by determining the expression of phospho-MEK1/2 and phospho-ERK1/2 in untreated cells and following a 24, 48, 72, 96 and 120 hours incubation with PrAg/LF using single-cell intracellular staining on flow cytometry.

A representative panel of 3 AML cell lines, namely ML2, HL60 and U937, was tested and the expression of phospho-MEK1/2 and phospho-ERK1/2 was related to how cells would react upon MAPK pathway inhibition under the effect of LF. Starting with phospho-MEK1/2, HL60 and ML2, sensitive cell lines, exhibited a decreased expression of phospho-MEK1/2 in the treated cells vs untreated ones at 24 and 48 hours after treatment. However, at 72, 96 and 120 hours, the expression of phospho-MEK1/2 increased significantly in the treated versus the nontreated samples in both cell lines (Figure 6 A, A', B, and B'). On the other hand, U937, a resistant cell line showed a comparable level of expression in treated and nontreated cells in all tested time points (Figure 6 C and C')

Staining for Phospho-ERK1/2 showed a decrease in expression in the treated cells vs untreated

ones at 24 and 48 hours after treatment in HL60 and ML2. This decrease was reversed after 72, 96 and 120 hours where phospho-ERK1/2 increased significantly in the treated versus the nontreated samples in both cell lines (Figure 7 A, A', B, and B'). Whereas levels of phospho-ERK1/2 in U937 were also comparable in treated and nontreated cells in all tested time points (Figure 7 C and C').

Interestingly, in HL60 cell line, and at later time points, starting at 72 hours, 2 populations of cells are there: the initial population that seems to fade, and another population that is actually appearing, and that's what is causing the increase in p-MEK1/2, p-ERK1/2 expression (Figure 6 A and A', Figure 7 A and A').

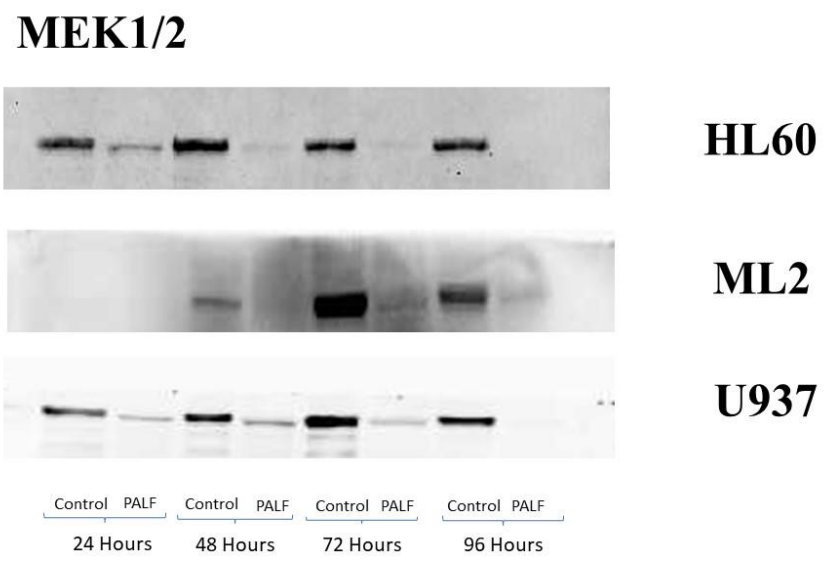


Figure 4: MEK Expression in HL60, ML2 and U937 in control untreated cells versus cells treated with wild type PALF at 24, 48, 72 and 96 hours.

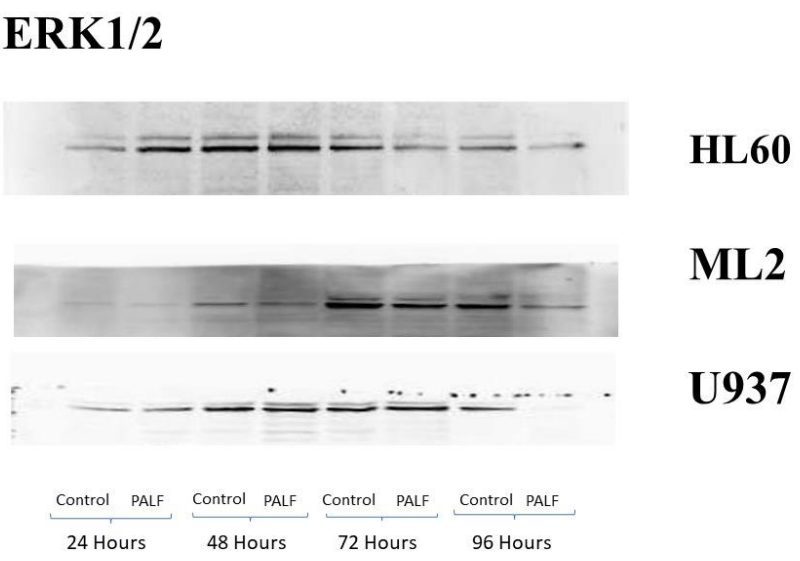
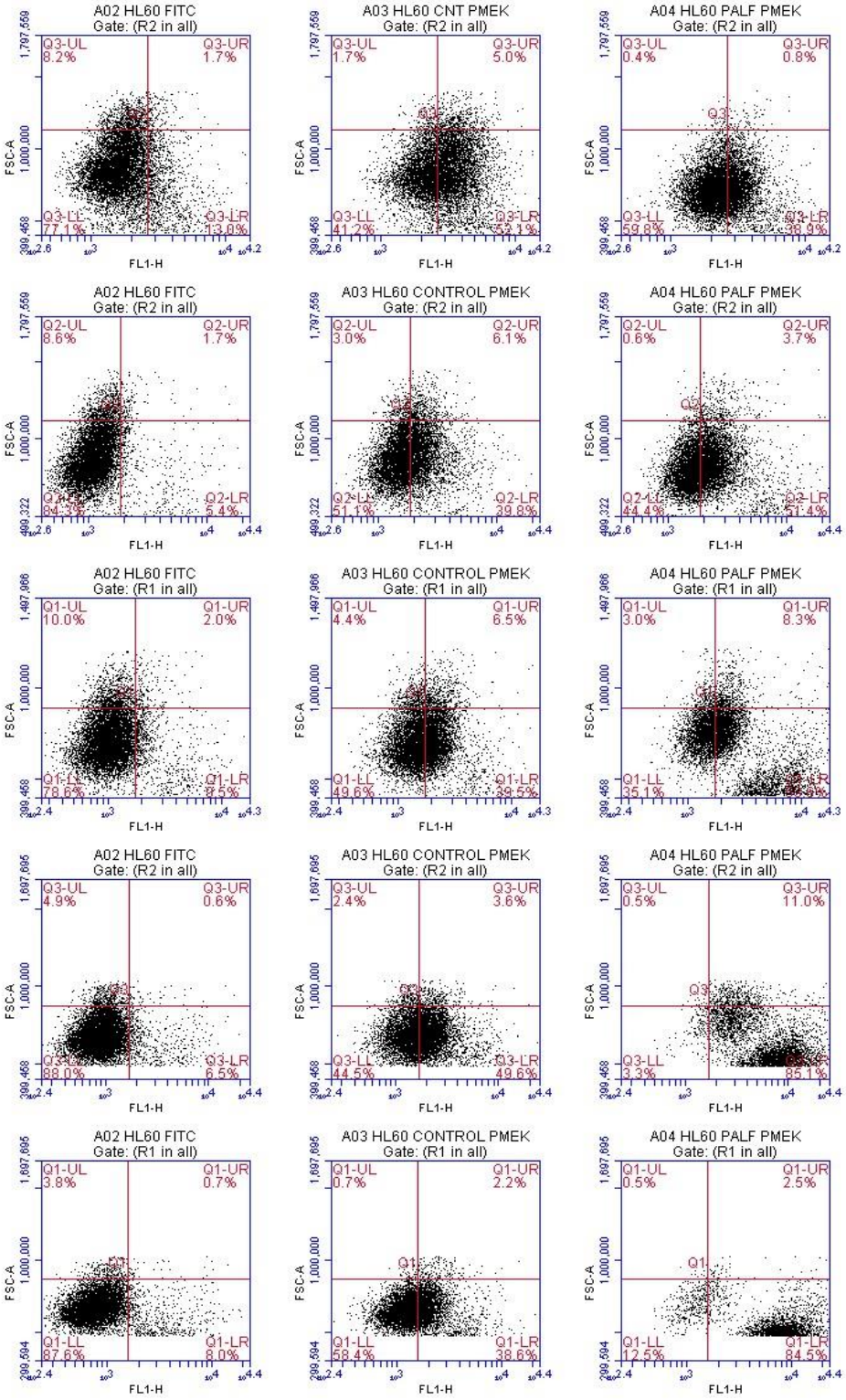


Figure 5: ERK Expression in HL60, ML2 and U937 in control untreated cells versus cells treated with wild type PALF at 24, 48, 72 and 96 hours.

A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Q3-UL 8.2%
Q3-UR 1.7%
Q3-LL 77.1%
Q3-LR 13.0%
A03 HL60 CNT PMEK
Gate: (R2 in all)
Q3-UL 1.7%
Q3-UR 5.0%
Q3-LL 41.2%
Q3-LR 52.1%
A04 HL60 PALF PMEK
Gate: (R2 in all)
Q3-UL 0.4%
Q3-UR 0.8%
Q3-LL 59.8%
Q3-LR 38.9%
A02 HL60 FITC
Gate: (R2 in all)
Q2-UL 8.6%
Q2-UR 1.7%
Q2-LL 84.3%
Q2-LR 5.4%
A03 HL60 CONTROL PMEK
Gate: (R2 in all)
Q2-UL 3.0%
Q2-UR 6.1%
Q2-LL 51.1%
Q2-LR 39.8%
A04 HL60 PALF PMEK
Gate: (R2 in all)
Q2-UL 0.6%
Q2-UR 3.7%
Q2-LL 44.4%
Q2-LR 51.4%
A02 HL60 FITC
Gate: (R1 in all)
Q1-UL 10.0%
Q1-UR 2.0%
Q1-LL 78.6%
Q1-LR 9.5%
A03 HL60 CONTROL PMEK
Gate: (R1 in all)
Q1-UL 4.4%
Q1-UR 6.5%
Q1-LL 49.6%
Q1-LR 39.5%
A04 HL60 PALF PMEK
Gate: (R1 in all)
Q1-UL 3.0%
Q1-UR 8.3%
Q1-LL 35.1%
A02 HL60 FITC
Gate: (R2 in all)
Q3-UL 4.9%
Q3-UR 0.6%
Q3-LL 88.0%
Q3-LR 6.5%
A03 HL60 CONTROL PMEK
Gate: (R2 in all)
Q3-UL 2.4%
Q3-UR 3.6%
Q3-LL 44.5%
Q3-LR 49.6%
A04 HL60 PALF PMEK
Gate: (R2 in all)
Q3-UL 0.5%
Q3-UR 11.0%
Q3-LL 3.3%
85.1%
A02 HL60 FITC
Gate: (R1 in all)
Q1-UL 3.8%
Q1-UR 0.7%
Q1-LL 87.6%
Q1-LR 8.0%
A03 HL60 CONTROL PMEK
Gate: (R1 in all)
Q1-UL 0.7%
Q1-UR 2.2%
Q1-LL 58.4%
Q1-LR 38.6%
A04 HL60 PALF PMEK
Gate: (R1 in all)
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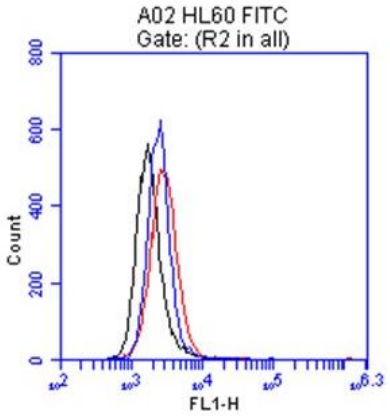
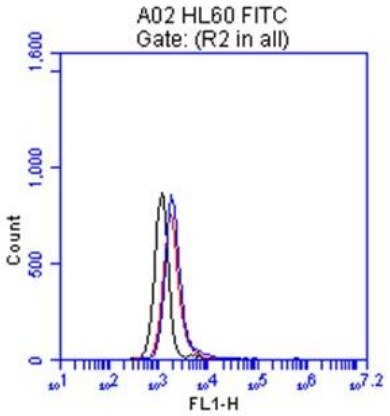
Q1-UR 2.5%
Q1-LL 12.5%
84.5%
FSC-A
FL1-H

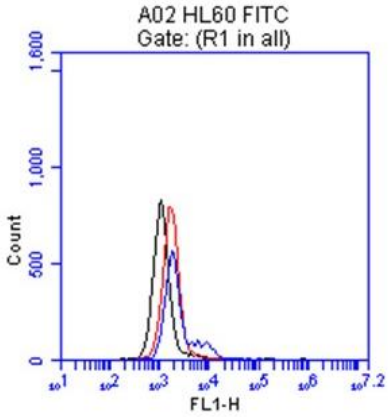
A'



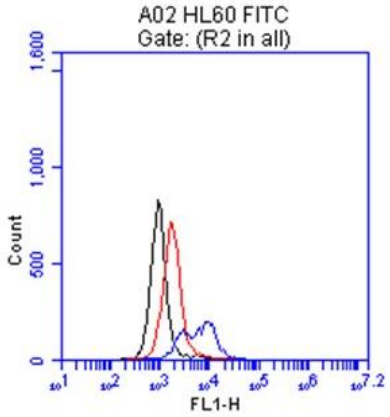
24 Hours



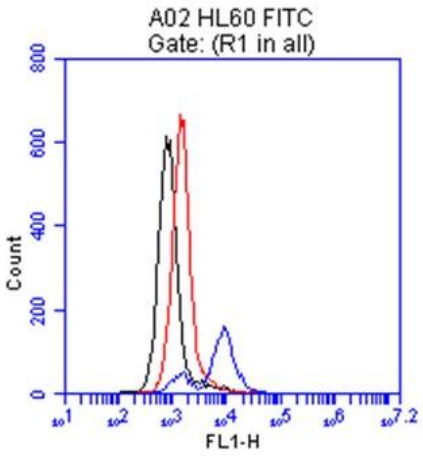
48 Hours



72 Hours

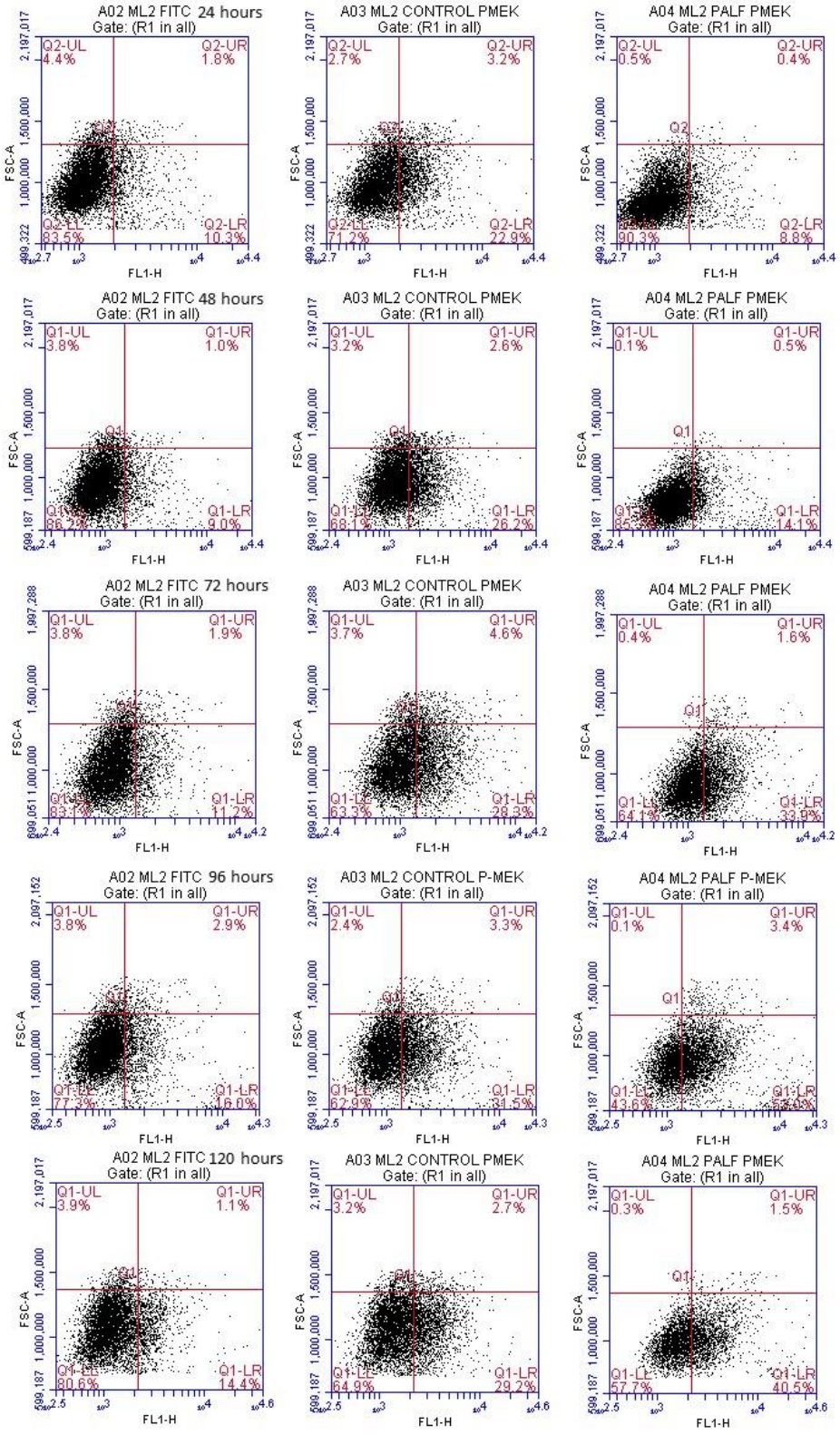


96 Hours



120 Hours

B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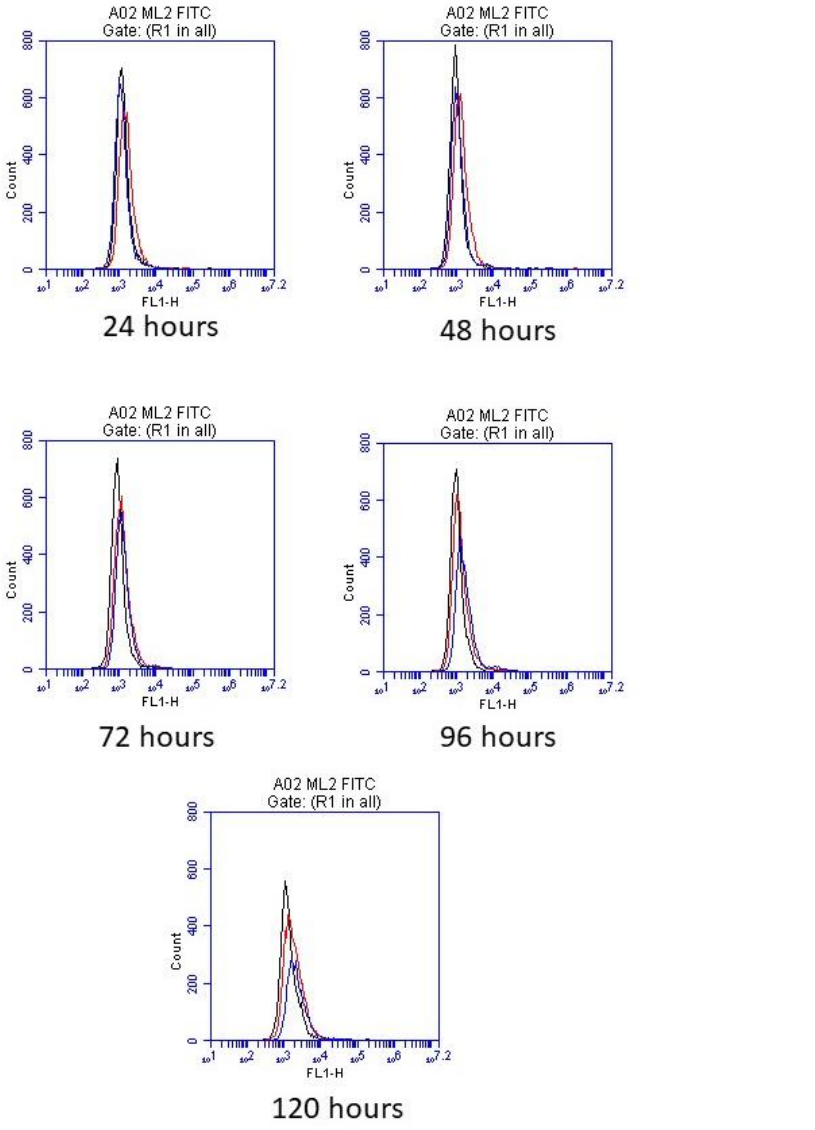
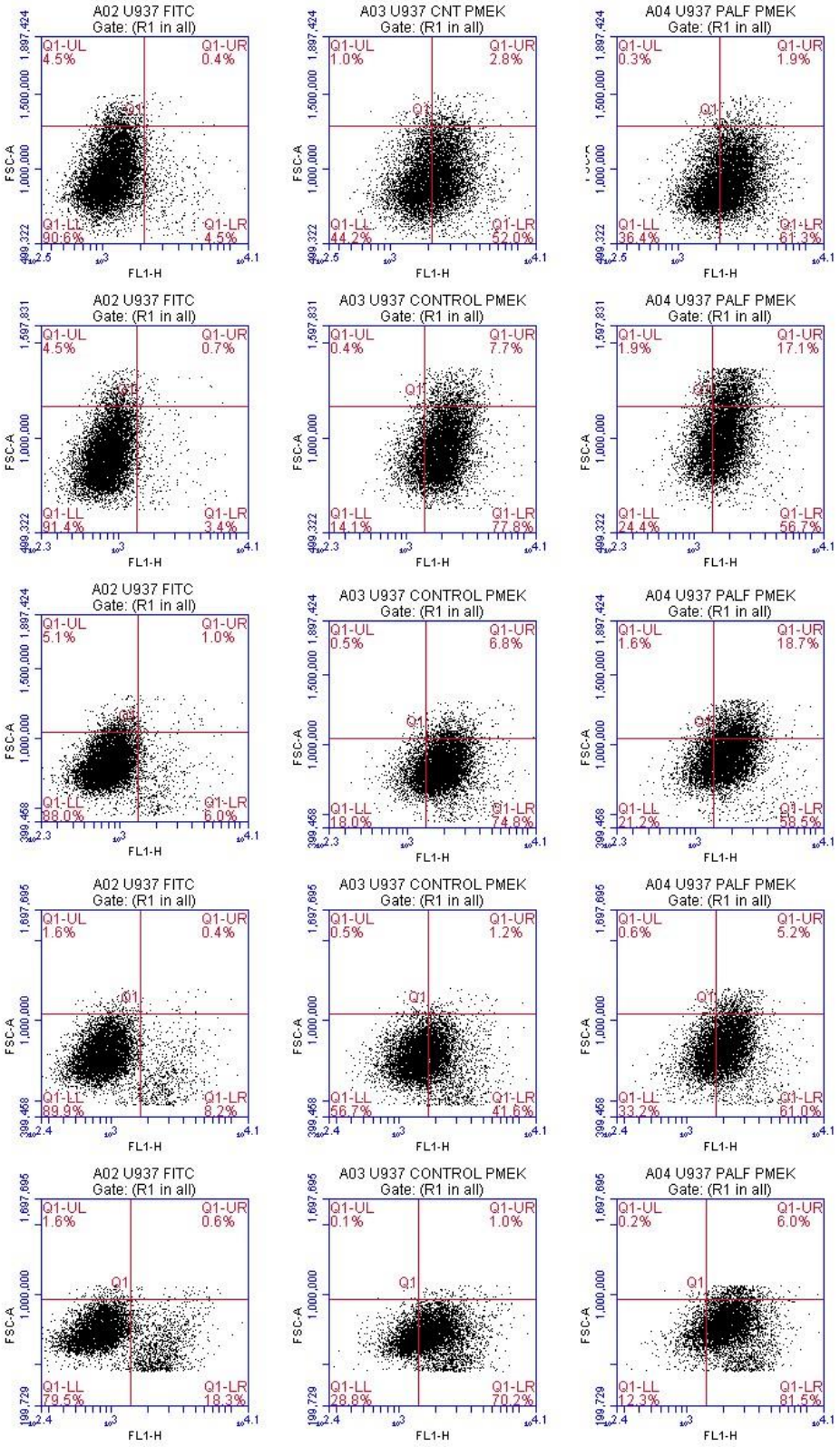
Gate: (R1 in all)
Q2-UL 4.4%
Q2-UR 1.8%
Q2-LL 83.5%
Q2-LR 10.3%
A03 ML2 CONTROL PMEK
Gate: (R1 in all)
Q2-UL 2.7%
Q2-UR 3.2%
Q2-LL 71.2%
Q2-LR 22.9%
A04 ML2 PALF PMEK
Gate: (R1 in all)
Q2-UL 0.5%
Q2-UR 0.4%
Q2-LR 8.8%
A02 ML2 FITC 48 hours
Gate: (R1 in all)
Q1-UL 3.8%
Q1-UR 1.0%
Q1-LR 9.0%
A03 ML2 CONTROL PMEK
Gate: (R1 in all)
Q1-UL 3.2%
Q1-UR 2.6%
Q1-LL 68.1%
Q1-LR 26.2%
A04 ML2 PALF PMEK
Gate: (R1 in all)
Q1-UL 0.1%
Q1-UR 0.5%
Q1-LR 14.1%
A02 ML2 FITC 72 hours
Gate: (R1 in all)
Q1-UL 3.8%
Q1-UR 1.9%
Q1-LR 11.2%
A03 ML2 CONTROL PMEK
Gate: (R1 in all)
Q1-UL 3.7%
Q1-UR 4.6%
Q1-LL 63.3%
Q1-LR 28.3%
A04 ML2 PALF PMEK
Gate: (R1 in all)
Q1-UL 0.4%
Q1-UR 1.6%
A02 ML2 FITC 96 hours
Gate: (R1 in all)
Q1-UL 3.8%
Q1-UR 2.9%
Q1-LL 77.3%
Q1-LR 16.0%
A03 ML2 CONTROL P-MEK
Gate: (R1 in all)
Q1-UL 2.4%
Q1-UR 3.3%
Q1-LL 62.9%
Q1-LR 31.5%
A04 ML2 PALF P-MEK
Gate: (R1 in all)
Q1-UL 0.1%
Q1-UR 3.4%
Q1-LL 43.6%
A02 ML2 FITC 120 hours
Gate: (R1 in all)
Q1-UL 3.9%
Q1-UR 1.1%
Q1-LL 80.6%
Q1-LR 14.4%
A03 ML2 CONTROL PMEK
Gate: (R1 in all)
Q1-UL 3.2%
Q1-UR 2.7%
Q1-LL 64.9%
Q1-LR 29.2%
A04 ML2 PALF PMEK
Gate: (R1 in all)
Q1-UL 0.3%
Q1-UR 1.5%
Q1-LL 57.7%
Q1-LR 40.5%
FSC-A
FL1-H

B’


A02 ML2 FITC
Gate: (R1 in all)
Count
FL1-H
24 hours
48 hours
72 hours
96 hours
120 hours
A02 ML2 FITC
A03 ML2 CONTROL PMEK
A04 ML2 PALF PMEK

C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Q1-LL 90.6%
Q1-LR 4.5%
A03 U937 CNT PMEK
Gate: (R1 in all)
Q1-UL 1.0%
Q1-UR 2.8%
Q1-LL 44.2%
Q1-LR 52.0%
A04 U937 PALF PMEK
Gate: (R1 in all)
Q1-UL 0.3%
Q1-UR 1.9%
Q1-LL 36.4%
Q1-LR 61.3%
A02 U937 FITC
Gate: (R1 in all)
Q1-UL 4.5%
Q1-UR 0.7%
Q1-LL 91.4%
Q1-LR 3.4%
A03 U937 CONTROL PMEK
Gate: (R1 in all)
Q1-UL 0.4%
Q1-UR 7.7%
Q1-LL 14.1%
Q1-LR 77.8%
A04 U937 PALF PMEK
Gate: (R1 in all)
Q1-UL 1.9%
Q1-UR 17.1%
Q1-LL 24.4%
Q1-LR 56.7%
A02 U937 FITC
Gate: (R1 in all)
Q1-UL 5.1%
Q1-UR 1.0%
Q1-LL 88.0%
Q1-LR 6.0%
A03 U937 CONTROL PMEK
Gate: (R1 in all)
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Q1-UR 6.8%
Q1-LL 18.0%
Q1-LR 74.8%
A04 U937 PALF PMEK
Gate: (R1 in all)
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Q1-UR 18.7%
Q1-LL 21.2%
Q1-LR 58.5%
A02 U937 FITC
Gate: (R1 in all)
Q1-UL 1.6%
Q1-UR 0.4%
Q1-LL 89.9%
Q1-LR 8.2%
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Gate: (R1 in all)
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Q1-UR 1.2%
Q1-LL 56.7%
Q1-LR 41.6%
A04 U937 PALF PMEK
Gate: (R1 in all)
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Q1-UR 5.2%
Q1-LL 33.2%
Q1-LR 61.0%
A02 U937 FITC
Gate: (R1 in all)
Q1-UL 1.6%
Q1-UR 0.6%
Q1-LL 79.5%
Q1-LR 18.3%
A03 U937 CONTROL PMEK
Gate: (R1 in all)
Q1-UL 0.1%
Q1-UR 1.0%
Q1-LL 28.8%
Q1-LR 70.2%
A04 U937 PALF PMEK
Gate: (R1 in all)
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Q1-UR 6.0%
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Q1-LR 81.5%
FSC-A
FL1-H

C’

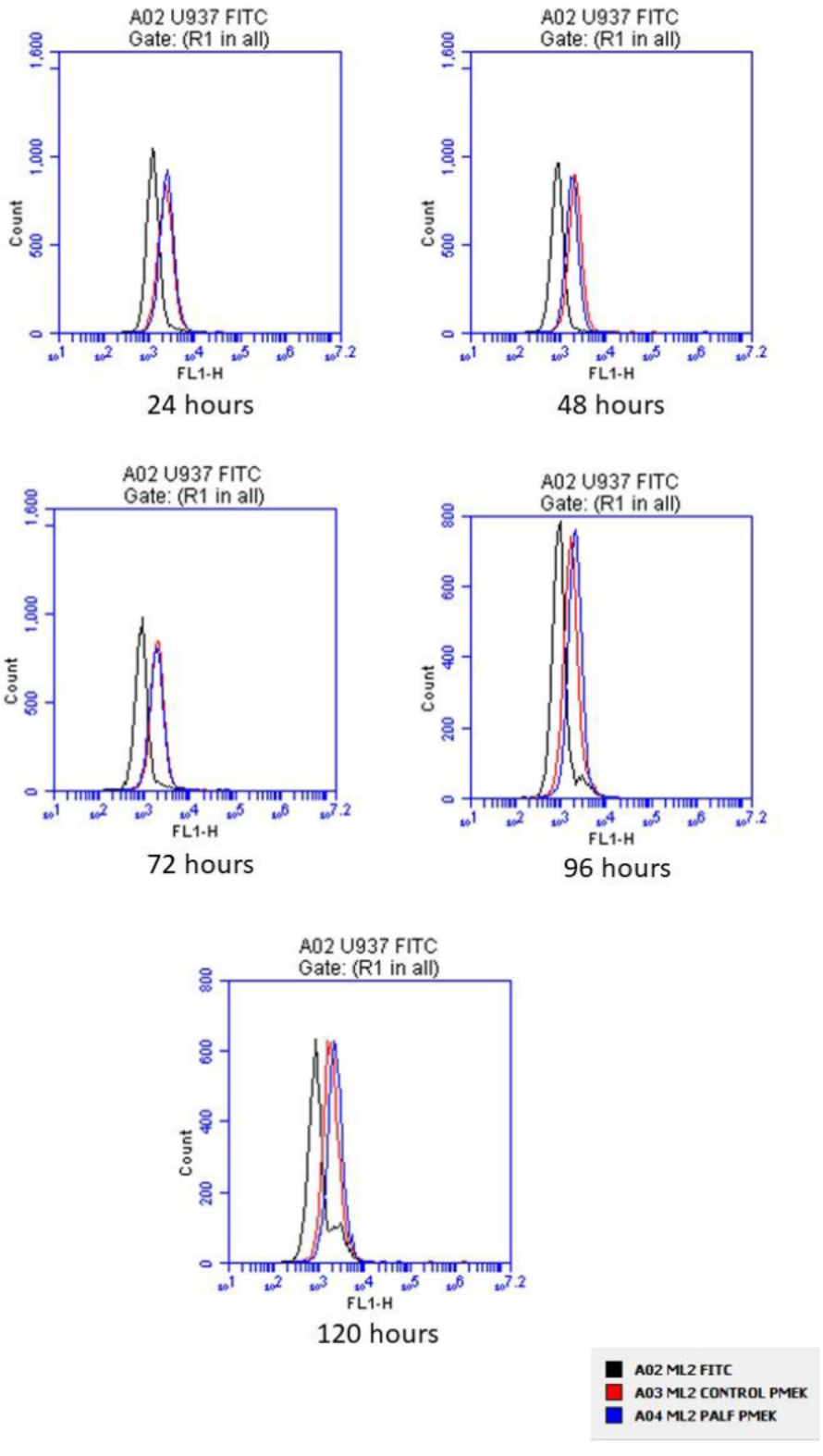
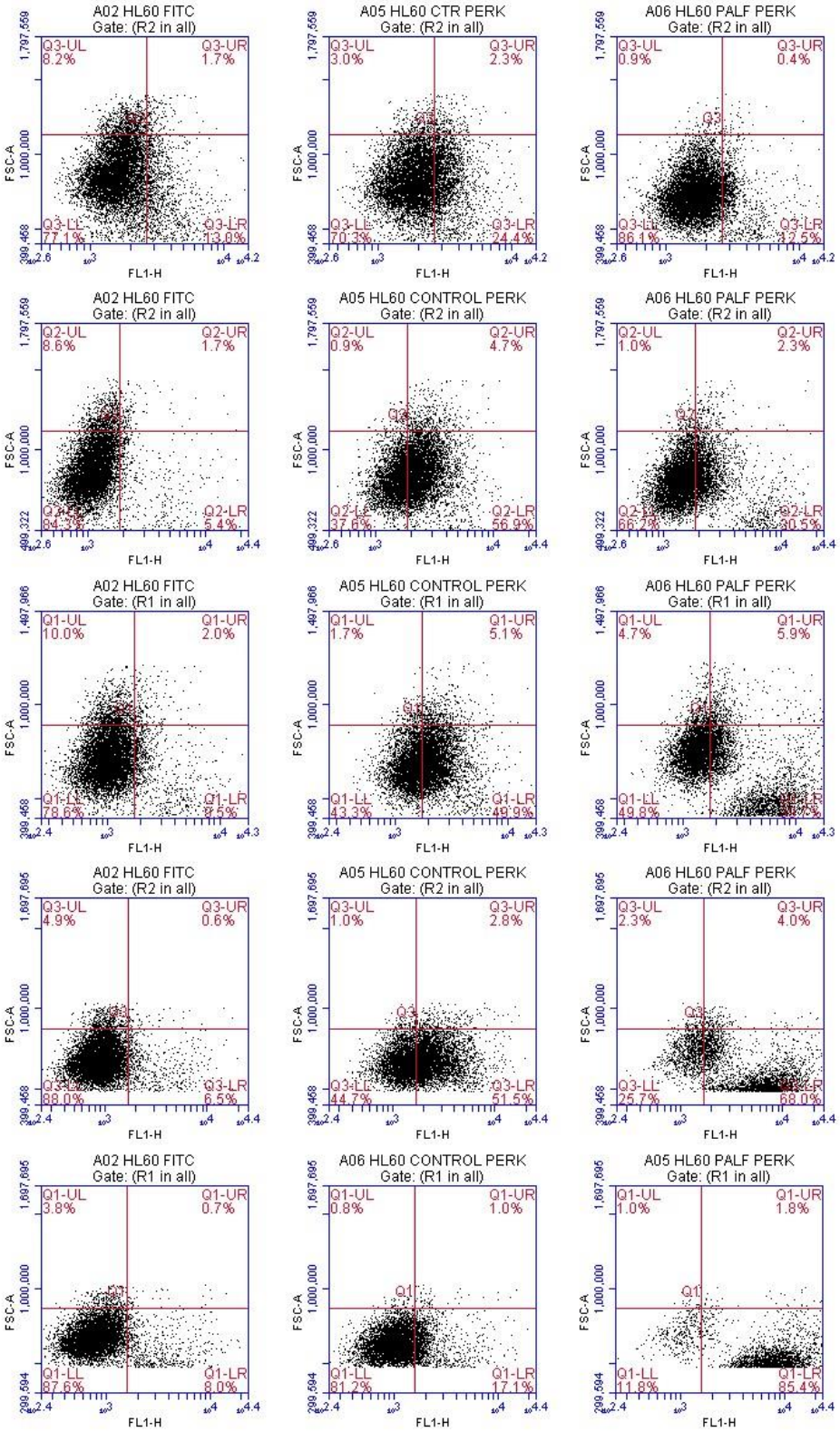


Figure 6: ERK Expression in HL60, ML2 and U937 in control untreated cells versus cells treated with wild type PALF at 24, 48, 72 and 96 hours.

A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Gate: (R2 in all)
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Q3-UR 1.7%
Q3-LL 77.1%
Q3-LR 13.0%
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Gate: (R2 in all)
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Q3-UR 2.3%
Q3-LL 70.3%
Q3-LR 24.4%
A06 HL60 PALF PERK
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Q3-UR 0.4%
Q3-LL 86.1%
Q3-LR 12.5%
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Q2-LR 56.9%
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Q2-LL 66.2%
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Gate: (R1 in all)
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Q1-LL 43.3%
Q1-LR 49.9%
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Gate: (R1 in all)
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Q3-LL 88.0%
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Q1-UR 0.7%
Q1-LL 87.6%
Q1-LR 8.0%
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Q1-LL 81.2%
Q1-LR 17.1%
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Gate: (R1 in all)
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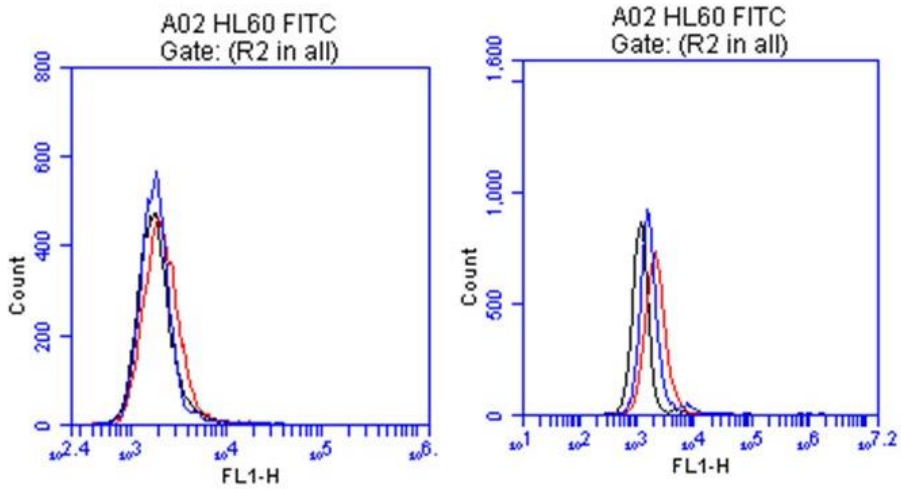
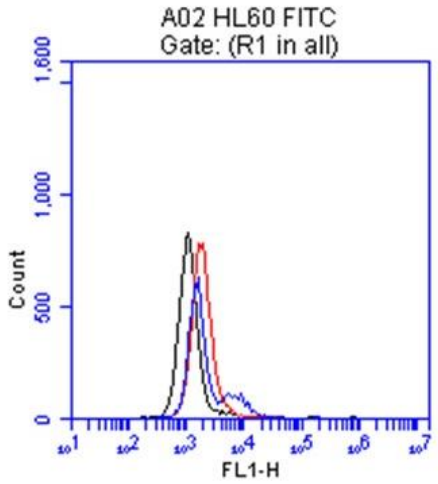
FSC-A
FL1-H

A'



24 Hours

48 Hours



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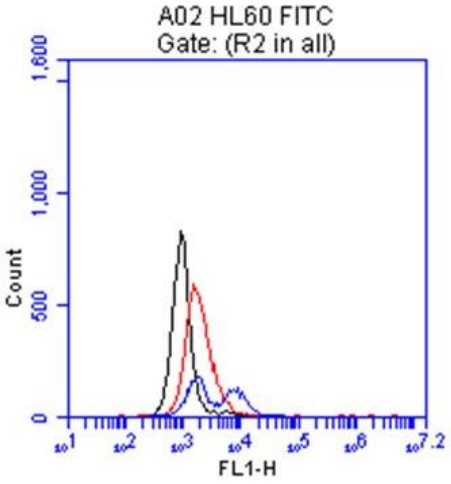
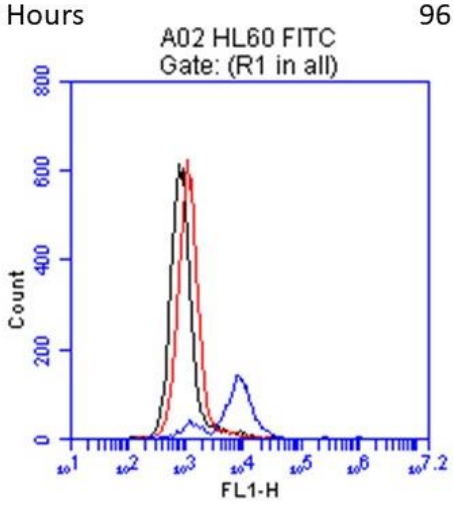
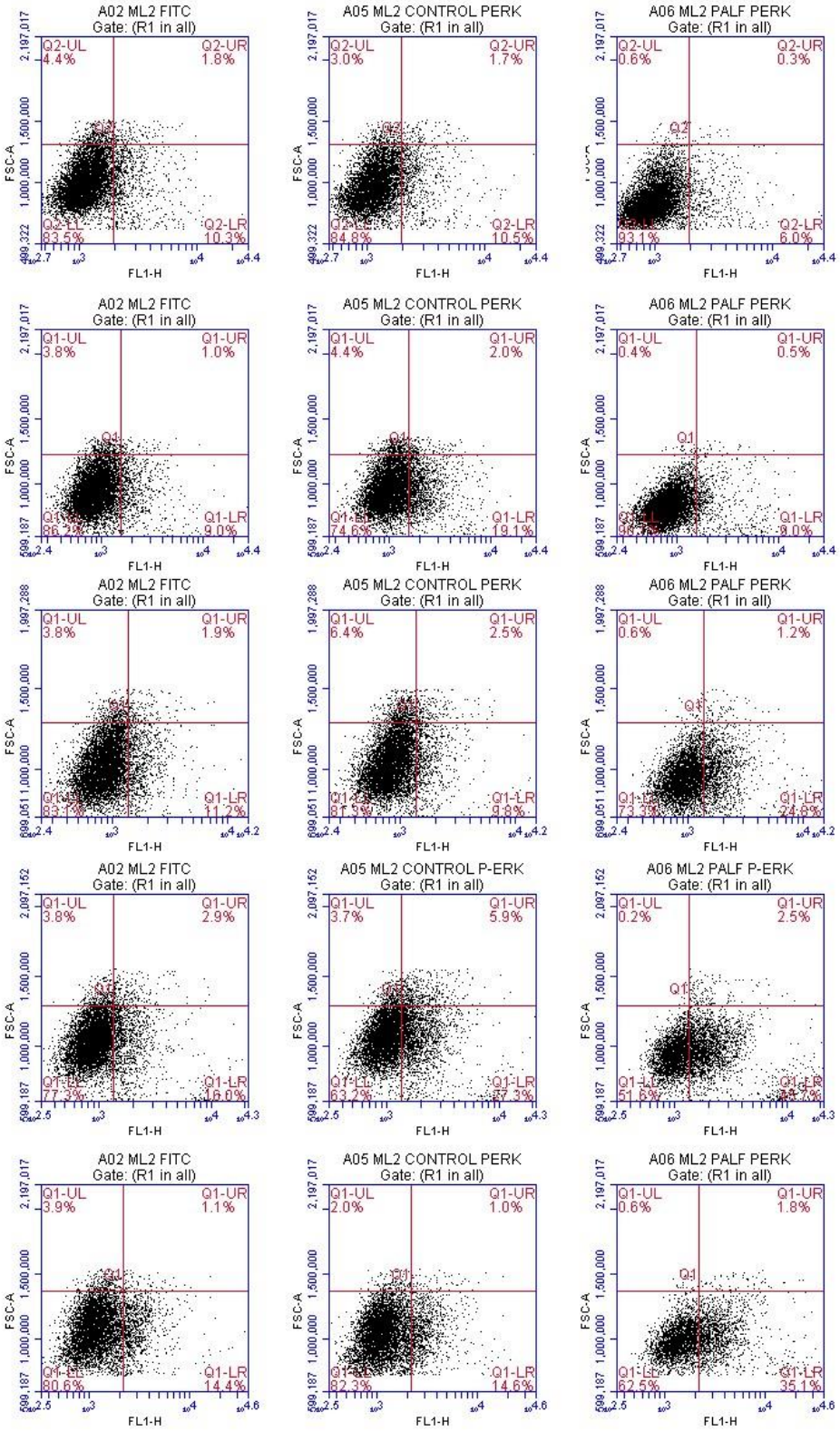


96 Hours



120 Hours

B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Gate: (R1 in all)
Q2-UL 3.0%
Q2-UR 1.7%
Q2-LL 84.8%
Q2-LR 10.5%
A06 ML2 PALF PERK
Gate: (R1 in all)
Q2-UL 0.6%
Q2-UR 0.3%
Q2-LL 93.1%
Q2-LR 6.0%
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Gate: (R1 in all)
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Q1-UR 1.0%
Q1-LR 9.0%
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Q1-LR 19.1%
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Gate: (R1 in all)
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Q1-UR 1.9%
Q1-LR 11.2%
A05 ML2 CONTROL PERK
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Q1-UR 2.5%
Q1-LR 9.8%
A06 ML2 PALF PERK
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Q1-UR 1.2%
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Q1-UR 2.9%
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Q1-LR 37.3%
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Gate: (R1 in all)
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Q1-UR 2.5%
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Gate: (R1 in all)
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Q1-LR 14.6%
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Gate: (R1 in all)
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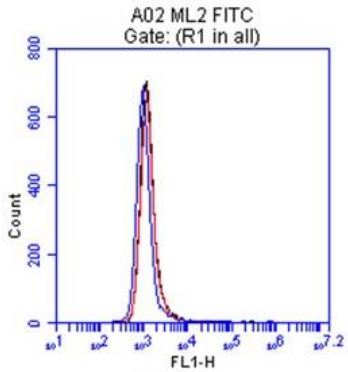
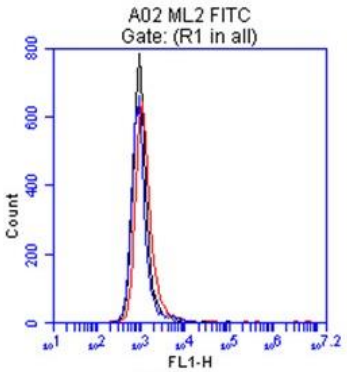
FSC-A
FL1-H

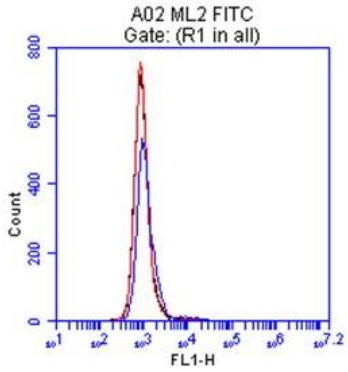
B’



24 hours



48 hours



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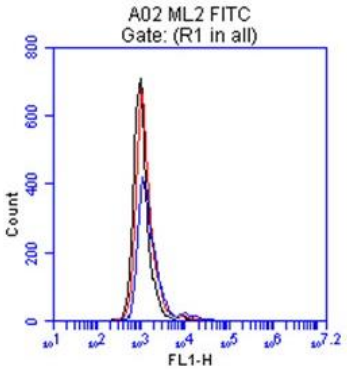
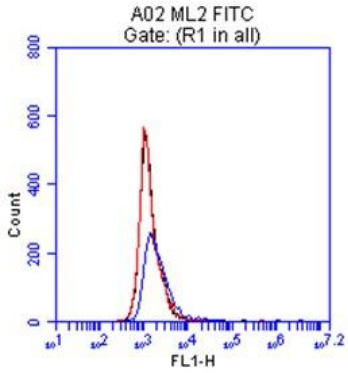
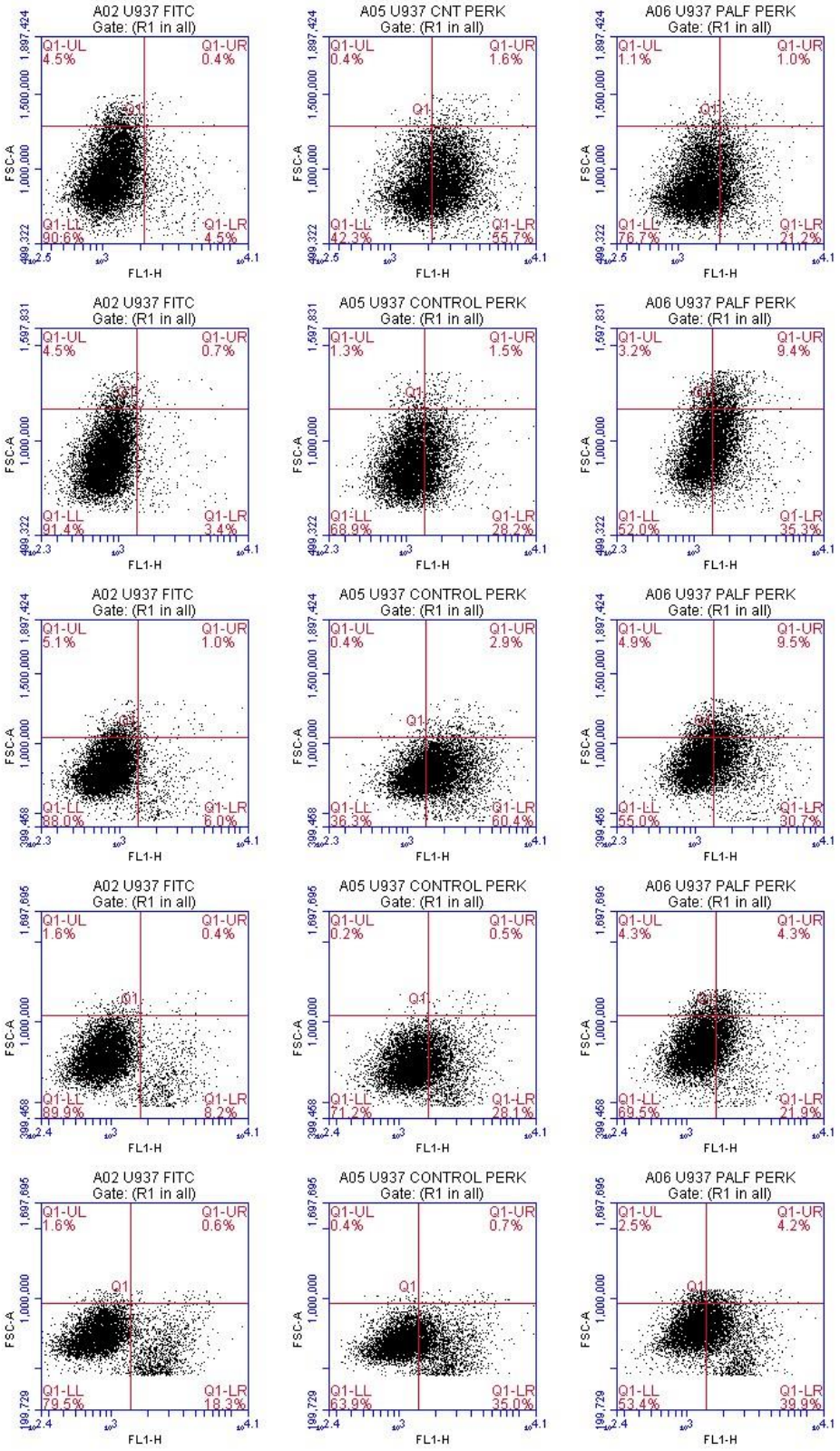


96 hours



120 hours

C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Q1-LR 4.5%
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Gate: (R1 in all)
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Q1-UR 1.6%
Q1-LL 42.3%
Q1-LR 55.7%
A06 U937 PALF PERK
Gate: (R1 in all)
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Q1-UR 1.0%
Q1-LL 76.7%
Q1-LR 21.2%
A02 U937 FITC
Gate: (R1 in all)
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Q1-UR 0.7%
Q1-LL 91.4%
Q1-LR 3.4%
A05 U937 CONTROL PERK
Gate: (R1 in all)
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Q1-LR 28.2%
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Gate: (R1 in all)
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Q1-LL 52.0%
Q1-LR 35.3%
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Q1-UR 1.0%
Q1-LL 88.0%
Q1-LR 6.0%
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Gate: (R1 in all)
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Q1-LL 36.3%
Q1-LR 60.4%
A06 U937 PALF PERK
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Q1-LL 55.0%
Q1-LR 30.7%
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Q1-LR 8.2%
A05 U937 CONTROL PERK
Gate: (R1 in all)
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Q1-UR 0.5%
Q1-LL 71.2%
Q1-LR 28.1%
A06 U937 PALF PERK
Gate: (R1 in all)
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Q1-UR 4.3%
Q1-LL 69.5%
Q1-LR 21.9%
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Gate: (R1 in all)
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Q1-UR 0.6%
Q1-LL 79.5%
Q1-LR 18.3%
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Gate: (R1 in all)
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Q1-UR 0.7%
Q1-LL 63.9%
Q1-LR 35.0%
A06 U937 PALF PERK
Gate: (R1 in all)
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Q1-UR 4.2%
Q1-LL 53.4%
Q1-LR 39.9%
FSC-A
FL1-H

C’

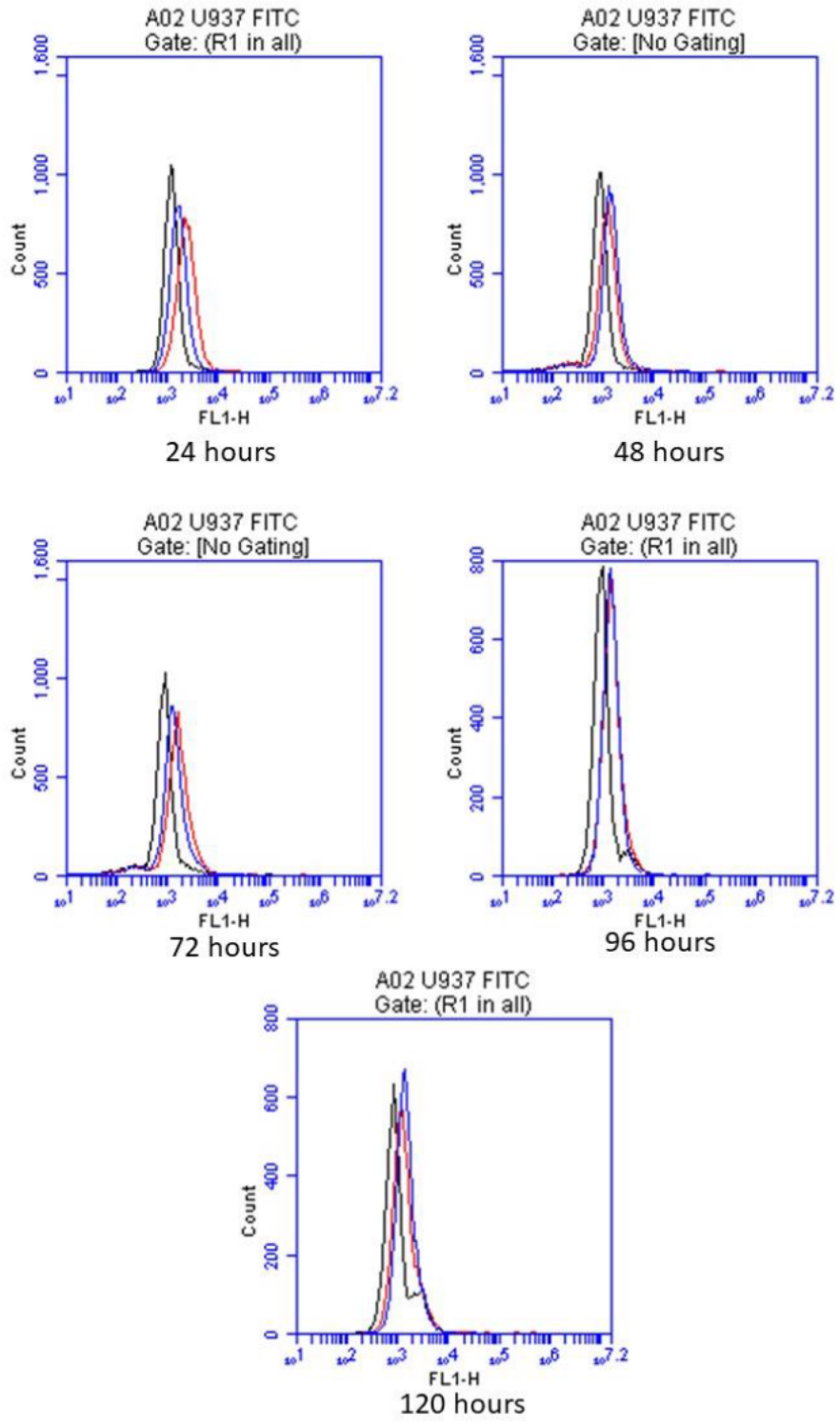


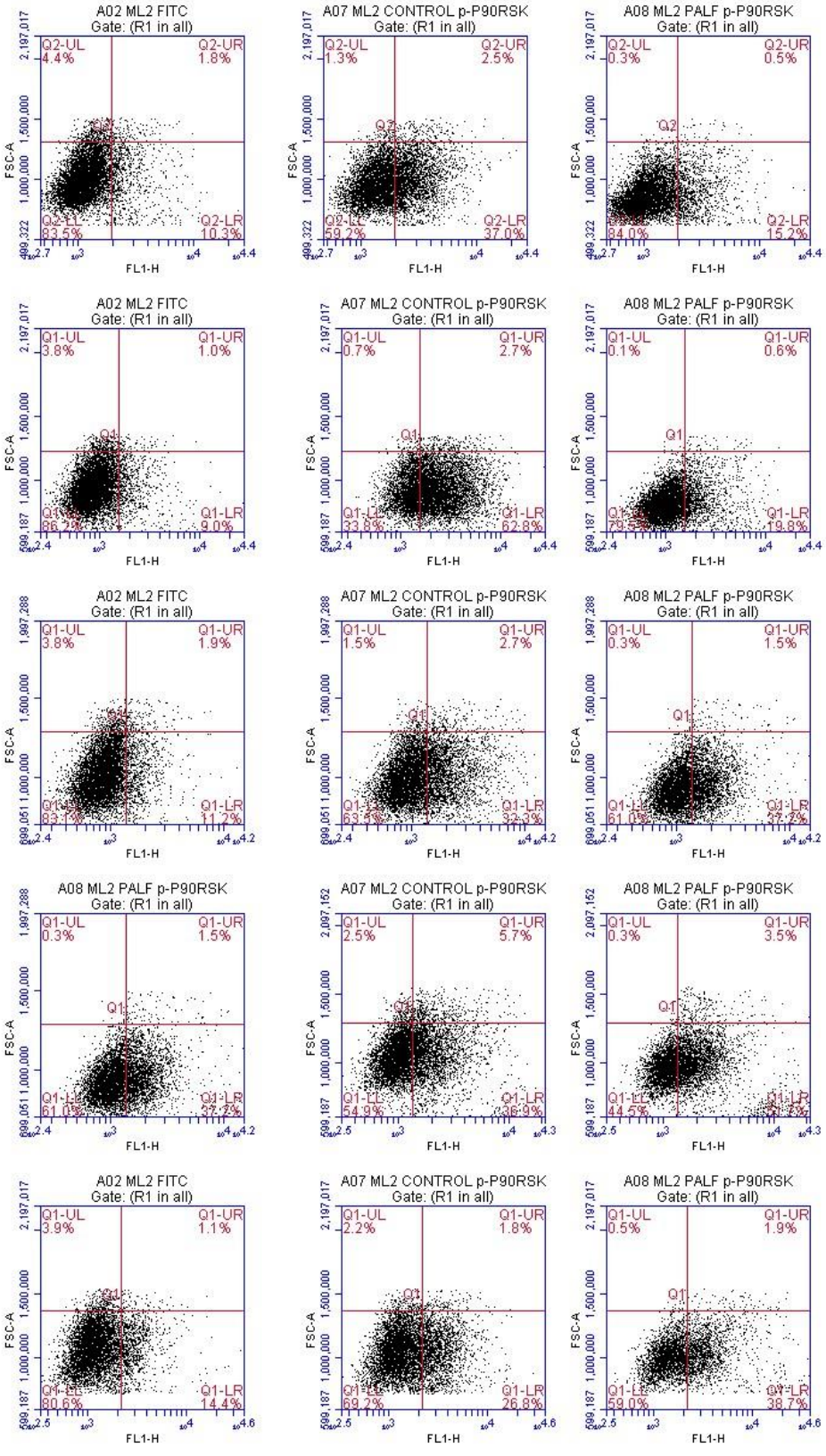
Figure 7: p-ERK expression in HL60 (A,A’), ML2 (B,B’),U937 (C,C’): from left to right: isotopic control (Black curve), control untreated (Red curve), treated with PrAgLF (Blue curve at 24, 48, 72, 96 and 120 hours post-treatment.

3.2 Effect of PrAg/LF on the Downstream Effectors of ERK:

Then, we examined the activation level of the MAPK pathway in AML cell lines by determining the expression of Phospho-p90RSK, a downstream effector of ERK in untreated cells and following a 24, 48, 72, 96 and 120 hours incubation with PrAg/LF using single-cell intracellular staining on flow cytometry.

As for phospho-p90RSK tested on ML2 only, intracellular staining showed a decrease in expression in the treated cells vs untreated ones at 24 and 48 hours after treatment, and an increase in expression after 72, 96 and 120 hours after treatment (Figure 8 A and A').

A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Q2-UR 1.8%
Q2-LL 83.5%
Q2-LR 10.3%
A07 ML2 CONTROL p-P90RSK
Gate: (R1 in all)
Q2-UL 1.3%
Q2-UR 2.5%
Q2-LL 59.2%
Q2-LR 37.0%
A08 ML2 PALF p-P90RSK
Gate: (R1 in all)
Q2-UL 0.3%
Q2-UR 0.5%
Q2-LL 84.0%
Q2-LR 15.2%
A02 ML2 FITC
Gate: (R1 in all)
Q1-UL 3.8%
Q1-UR 1.0%
Q1-LL 86.2%
Q1-LR 9.0%
A07 ML2 CONTROL p-P90RSK
Gate: (R1 in all)
Q1-UL 0.7%
Q1-UR 2.7%
Q1-LL 33.8%
Q1-LR 62.8%
A08 ML2 PALF p-P90RSK
Gate: (R1 in all)
Q1-UL 0.1%
Q1-UR 0.6%
Q1-LR 19.8%
A02 ML2 FITC
Gate: (R1 in all)
Q1-UL 3.8%
Q1-UR 1.9%
Q1-LL 83.1%
Q1-LR 11.2%
A07 ML2 CONTROL p-P90RSK
Gate: (R1 in all)
Q1-UL 1.5%
Q1-UR 2.7%
Q1-LL 63.5%
Q1-LR 32.3%
A08 ML2 PALF p-P90RSK
Gate: (R1 in all)
Q1-UL 0.3%
Q1-UR 1.5%
Q1-LL 61.0%
Q1-LR 37.2%
A08 ML2 PALF p-P90RSK
Gate: (R1 in all)
Q1-UL 0.3%
Q1-UR 1.5%
Q1-LL 61.0%
Q1-LR 37.2%
A07 ML2 CONTROL p-P90RSK
Gate: (R1 in all)
Q1-UL 2.5%
Q1-UR 5.7%
Q1-LL 54.9%
Q1-LR 36.9%
A08 ML2 PALF p-P90RSK
Gate: (R1 in all)
Q1-UL 0.3%
Q1-UR 3.5%
Q1-LL 44.5%
Q1-LR 52.5%
A02 ML2 FITC
Gate: (R1 in all)
Q1-UL 3.9%
Q1-UR 1.1%
Q1-LL 80.6%
Q1-LR 14.4%
A07 ML2 CONTROL p-P90RSK
Gate: (R1 in all)
Q1-UL 2.2%
Q1-UR 1.8%
Q1-LL 69.2%
Q1-LR 26.8%
A08 ML2 PALF p-P90RSK
Gate: (R1 in all)
Q1-UL 0.5%
Q1-UR 1.9%
Q1-LL 59.0%
Q1-LR 38.7%
FSC-A
FL1-H

A'

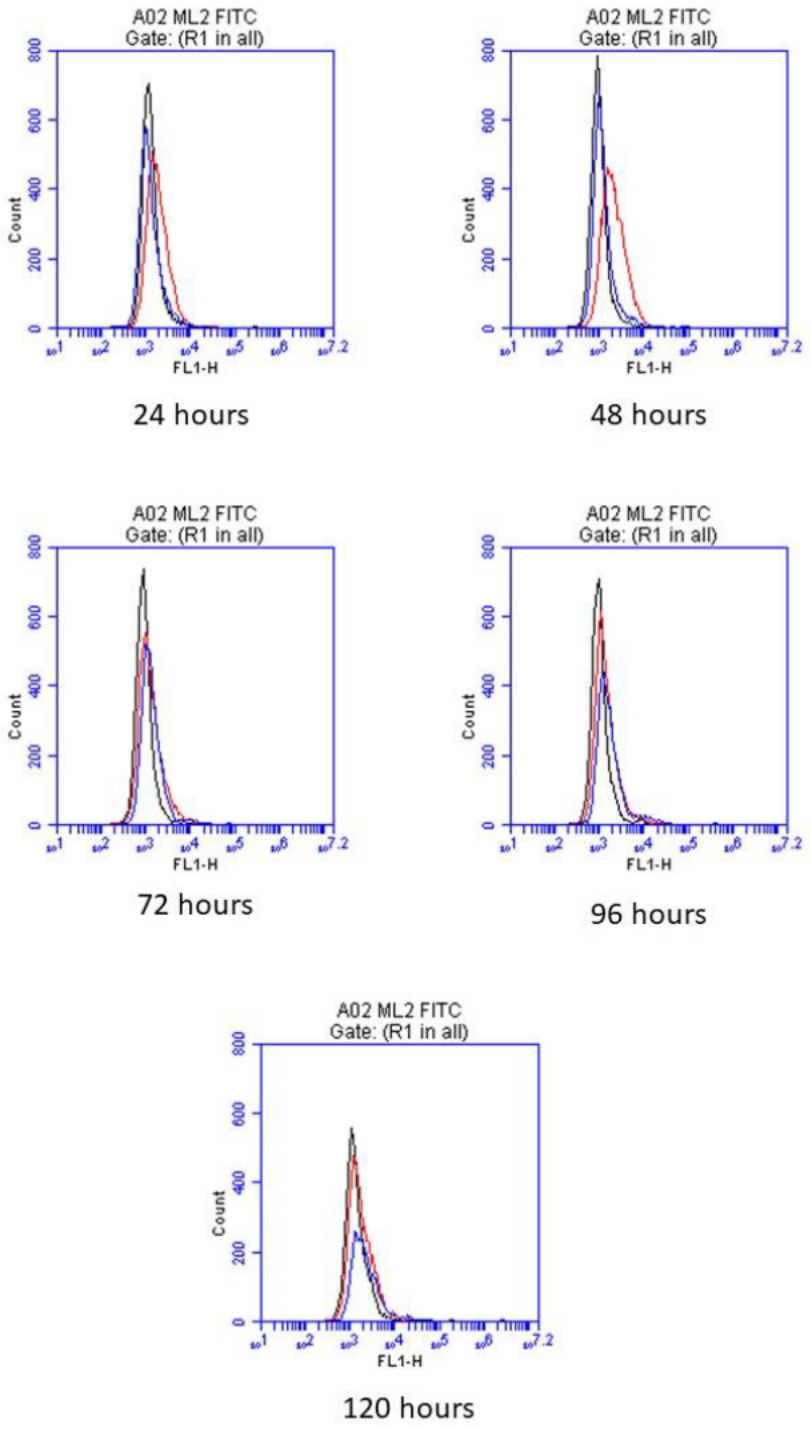

Figure 8: p-p90RSK expression in ML2 (A,A'): from left to right: isotopic control (Black curve), control untreated (Red curve), treated with PrAgLF (Blue curve at 24, 48, 72, 96 and 120 hours post-treatment.

3.3 Effect of Anthrax Lethal Toxin on Autophagy:

To determine whether autophagy is activated as a result of MAPK pathway inhibition due to treatment with PrAg/LF, we examined the expression of LC3-2 in AML cell lines, U937 and ML2 after treatment with PrAg/LF for 24, 48, 72, 96 and 120 hours. Conditions were control untreated cells, cells treated with PrAg/LF, cells treated with PrAg/LF and CQ, cells treated with CQ alone.

Western blots of LC3 1 and 2 show that expression of LC3-2 is more in PrAg/LF + CQ cells than in cells treated with PrAg/LF alone or CQ alone and this shows an increase in the flux of autophagy, the increase seems to be at 24 and 48 hours but it decreases at later time points for the sensitive cell line which is ML2 in this case. Similarly, U937 western blots show an increase in the flux of autophagy where LC3-2 is expressed more in cells treated with PrAg/LF + CQ, compared to cells treated with either PrAg/LF or CQ alone. This increase however seems to be more persistent, it lasts to later time points.

Note that the bar graphs inserted below are just a bar graph representation of the LC3 results with no statistical significance analysis.

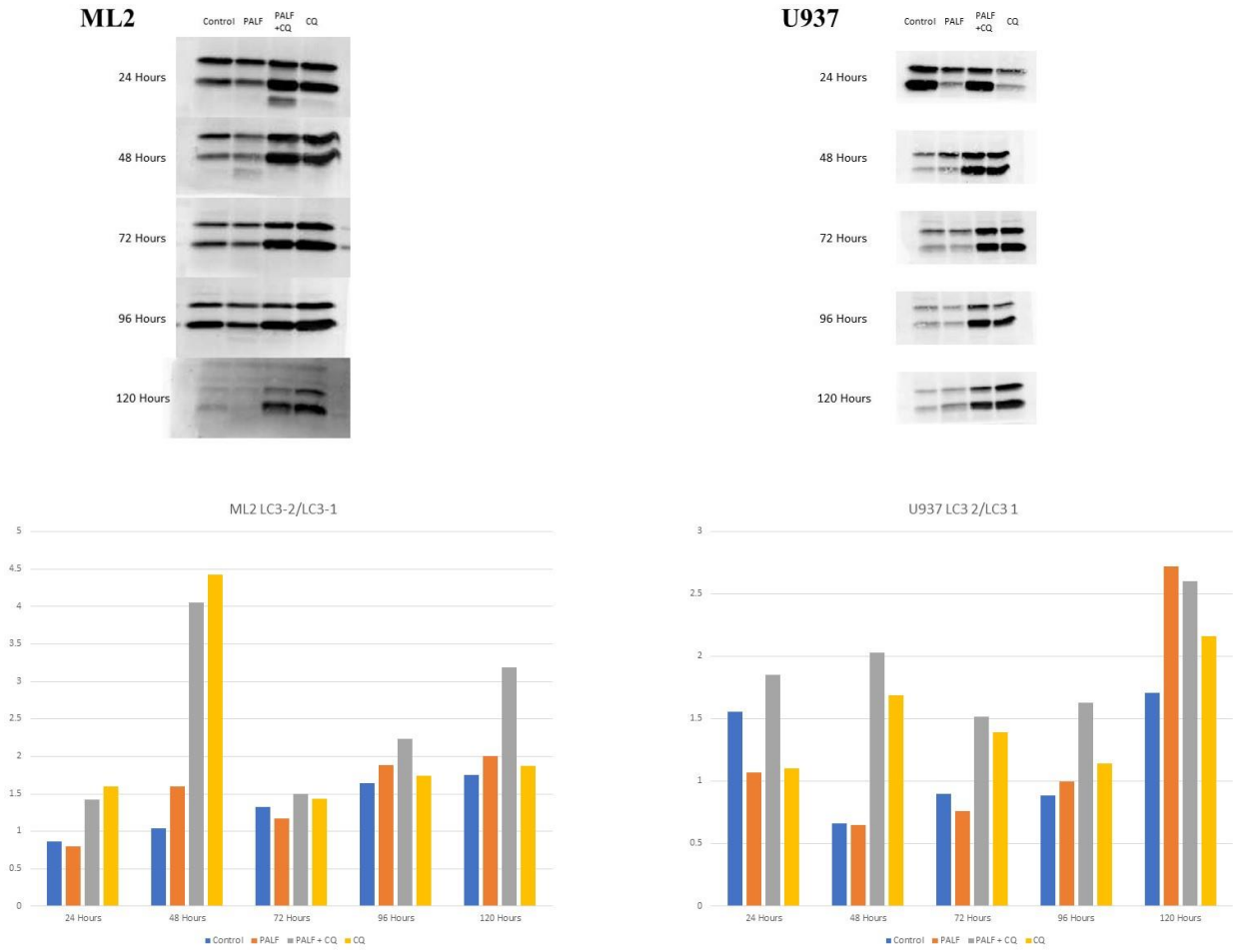

Figure 9: LC3 cleavage in ML2 and U937 cells at 24, 48, 72, 96 and 120 h following treatment on western blot

Chapter Four

Discussion

seven out of eleven (around 64%) AML cell lines demonstrated a cytotoxic response to LeTx and this cytotoxicity was similar to that induced by U0126, a MEK1/2 inhibitor, proving that this cytotoxic response caused by anthrax lethal toxin is due to inhibition of MEK1/2-ERK1/2 branch of MAPK pathway (Kassab et al., 2013).

However, the reason why some cells are showing sensitivity to anthrax lethal toxin while others seem to survive this drug is still unknown. In this study, we attempt to investigate the mechanism of cell death in sensitive cell lines and hence the mechanism of resistance in resistant ones.

First, we confirmed the effect of anthrax lethal toxin on MEK using western blotting. Western blots show that anthrax lethal toxin degraded MEK in treated cells vs control nontreated cells as early as 24 hours after treatment in all tested cell lines, both sensitive and resistant.

Then, we determined the activity of the Ras-Raf-MEK1/2-ERK1/2 pathway in AML cell lines by assessing phospho-MEK1/2, phospho-ERK1/2 and phospho-p90RSK levels using single cell intracellular staining and comparing it to the cytotoxic response of these cells to the LF-mediated inhibition of the MAPK pathway.

For sensitive cell lines such as ML2 and HL60, the pattern of expression is common for all phosphorylated forms of the members of the MAPK pathway. The treatment with anthrax lethal toxin caused a decrease in expression of phospho-MEK1/2, phospho-ERK1/2 and phospho-p90RSK up to 48 hours following treatment. This effect was reversed at 72 hours and more for the 3 phosphorylated forms. Note that in addition to this, a biphasic response in HL60 is observed: two populations of cells are there: the initial population that seems to fade with time, and another population that is starting to appear at 72 hours. That means that we have a sub

population of cells within this sensitive cell line that is resistant and manages to increase its MEK, ERK phosphorylation and survives the PrAg/LF treatment. The interpretation of this biphasic response is not really known and is to be investigated more extensively.

However, this pattern was not observed in U937 where LF mediated MAPK pathway inhibition did not induce cytotoxicity. In all time points, the levels of expression of phospho-MEK1/2 and phospho-ERK1/2 are comparable with no significant decrease or increase, between the treated and untreated cells.

Adding these all together, western blot is showing that we are actually degrading MEK in all these cell lines. But this is not that simple. We are degrading MEK but in flow we see that MEK is coming back at later time points in sensitive cells and in resistant cells we have no effect on p-MEK, p-ERK and p-p90RSK.

Although p-MEK, p-ERK and p-p90RSK are coming back and increasing, this does not seem to affect cytotoxicity. The interpretation might be that when MEK starts degrading due to LF, the cell is going to respond by increasing the expression of MEK. It might be that at 24 and 48 hours PALF is degrading MEK, and by that time the cell had not have time to make more MEK and then by 72 hours, and when LF starts decreasing, cells are making more MEK allowing it to recover. But would that affect the viability of the cell? We don't think so because the cells will die anyways because they won't survive the first 48 hours of MAPK pathway inhibition. Note that the reason why this effect is not seen in western blots is because flow cytometry is a single cell analysis that is much more sensitive than western blotting.

In conclusion, even though we are degrading MEK in all these cell lines, their response seems to vary indicating that we have sensitive and resistant cell lines. This response must be studied and investigated to determine the mechanism of cell death in sensitive cell lines and hence the

mechanism of resistance in the resistant ones. In sensitive cells, the degradation of MEK, at least in early time points, suppresses p-MEK, p-ERK and p-p90RSK, while in resistant cell lines, such as U937, the degradation of MEK does not suppress these at any time point explaining why these cells are resistant. This indicates that Ras-Raf-MEK1/2-ERK1/2 pathway is affected differently in sensitive versus resistant cell lines and this may explain the difference in susceptibility of AML cell lines to anthrax lethal toxin.

As for autophagy, different AML cells lines, both sensitive and resistant showed activation of autophagy following treatment with PrAg/LF. This was proven by flow cytometry analysis for autophagosome formation (previous data) and western blot analysis for LC3, an autophagy marker. However, more data is needed to confirm that and confirm the timeline of autophagy activation and the impact of autophagy activation on the cytotoxic response of AML cells.

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