ALL-TRANS RETINOIC ACID AND ARSENIC TRIOXIDE INDUCE LASTING DIFFERENTIATION AND DEMETHYLATION OF TARGET GENES IN ACUTE PROMYELOCYTIC LEUKEMIA

by

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Submitted in partial fulfillment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, NS
August 2018

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ABSTRACT

Acute promyelocytic leukemia (APL) patients treated with all-trans retinoic acid (ATRA) experience relapse, while patients treated with ATRA and arsenic trioxide (ATO) are often relapse-free. To understand combination therapy, we compared the effects of ATRA, ATO or the combination, on NB4 APL cells during treatment versus 96h post treatment termination. After treatment termination, cells treated with ATRA or ATO reverted to non-differentiated cells, while combination-treated cells remained differentiated. ATRA and combination treatment induced similar levels of target genes during treatment; however, post treatment termination, combination-treated cells retained higher levels of target genes (e.g., transglutaminase 2 and retinoic acid receptor beta). We quantified enrichment of histone modifications by chromatin immunoprecipitation, and CpG methylation by bisulfite-pyrosequencing. While ATRA and combination treatment induced similar histone acetylation enrichment, only combination treatment reduced CpG methylation of target genes. Therefore, DNA methylation changes is associated with lasting differentiation and gene expression changes induced by combination treatment.

LIST OF ABBREVIATIONS USED

ALL	acute lymphocytic leukemia
AML	acute myeloid leukemia
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
APL	acute promyelocytic leukemia APL
WBC	white blood cell
ATRA	all-trans retinoic acid
DIC	disseminated intravascular coagulation
TF	tissue factor
CP	cancer procoagulant
PAI-1	plasminogen activator inhibitor 1
MP	microparticles
PML	promyelocytic leukemia
RARα	retinoic acid receptor α
PML-RARα	promyelocytic leukemia-retinoic acid receptor-alpha protein
RXR	RAR-retinoid-X-receptor
RARE	retinoic acid response elements
HDAC	histone deacetylases
PML-NB	PML nuclear bodies
FISH	florescent *in situ* hybridization
PLZF	promyelocytic leukemia zinc finger
STAT5b	signal transducer and activator of transcription 5b
5-mC	5-methylcytosine
DMNT	DNA methyl transferases
CpG	cytosine–guanine dinucleotide
TET	ten-eleven translocation
HMT	histone methyltransferases
H3K9me3	tri-methylation of lysine 9 on histone 3
PRC1/PRC2	polycomb repressive complex 1 and 2
H3M27me3	tri-methylation of lysine 27 on histone
HAT	histone acetyltransferase
H3K9ac	acetylation of lysine 9 of histone 3
CR	complete remission
MPO	myelodoperoxidase
C/EBP	CCAAT/enhancer binding protein
ATO	arsenic trioxide
RRBS	reduced representation bisulfite sequencing
LINE-1	long interspersed nucleotide element 1
GM-CSF	granulocyte-macrophage stimulating factor
ATCC	american type culture collection

ACKNOWLEDGEMENTS

Firstly, I would like to thank Dr. Paola Marcato for her guidance and support over the past 4 years as I progressed from an experiential learning student to a graduate student in her laboratory. Dr. Marcato demonstrated both endless patience in training me and belief in my ability to accomplish everything I have done in this program. Her dedication to research and the people around her has greatly inspired my work inside and outside of the laboratory.

I would also like to thank the members of my supervisory committee, Drs. David Waisman and Jason Berman whose knowledge, suggestions and support was extremely helpful in the preparation and completion of this thesis. I would like to thank Dejan Vidovic who was a steadfast friend in and out of the laboratory where we worked together for the past 4 years. I would also like to thank Cheryl Dean and Dr. Ian Weaver for their contribution to preparing the manuscript this thesis produced.

Finally, I want to thank my friends and family who were there for me throughout this whole experience and all the challenges and highlights it entailed. I am grateful for this great learning opportunity as I move forward with the next step in my life.

CHAPTER 1 INTRODUCTION

1.10 The stem cells of Leukemia

Cancer is a malignancy characterized by the inappropriate proliferation of cells in an uncontrolled manner. Leukemia is a subtype of cancer specifically involving the cells of the blood and currently affects over 20,000 Canadians[1]. Together, with other blood cancers such as lymphoma and myelomas, these hematological malignancies make up the third leading cause of cancer associated death of men and the fourth leading cause of women in Canada (Table 1)[1].

Leukemia originates from hematopoietic stem cells which are a blood specific type of stem cell[2]. Stem cells are a rare class of cells considered the "primary cells" of the body and are defined by several fundamental properties. Firstly, stem cells are pluripotent and can differentiate into the many different lineages of specialized cell types that make up the body[3]. The stem cell begins at an immature undifferentiated stage – in the presence of activating signals such as chemical growth factors or direct contact with other cells, the stem cell responds by undergoing differentiation into a cell fate dictated by the activating signals present[4]. Depending on the cell fate, there are generally multiple intermediate stages the stem cell transiently differentiates into before it develops into the final mature cell type[5]. However, as the stem cell proceeds through the differentiation process, it gradually loses its pluripotency property and becomes permanently changed at its terminal stage.

The other fundamental property of stem cells is their ability to self-renew indefinitely through continuous replication and division[5]. This stem cell property is especially important to maintain the populations of both stem cells and differentiated

Table 1. Incidence rate of new blood cancer cases in Canada in 2016.

Hematological Malignancy	Incidence
Lymphoma	9,000 cases
Leukemia • ALL: 5% • AML: 24% • CLL: 44% • CML:12 • Others: 15%	5,900 cases
Myelodysplastic syndrome	3,850 cases
Myeloma	2,700 cases
Polycythemia vera (PV)	400 cases
Essential (primary) thrombocythemia (ET/PT)	290 cases
Myelofibrosis	200 cases

cells. Together, these properties enable stem cells to replenish all the different types of cells the body requires to function.

There are several classes of cell-type specific stem cells including hematopoietic stem cells, mesenchymal stem cells and epithelial stem cells. Hematopoietic stem cells originate in the bone marrow or peripheral blood and differ slightly from pluripotent stem cells – these stem cells are more limited than pluripotent stem cells in the types of cells they can differentiate into, therefore are considered multipotent stem cells[6]. Specifically, hematopoietic stem cells can differentiate into the lymphoid or myeloid lineages, each with their own distinct precursors and terminal products[6]. To differentiate into the lymphoid lineage, the stem cell becomes a common lymphoid progenitor, and terminally differentiate into lymphocytes important for immune function[6]. Specifically, the class of lymphocytes consist of B cells, T cells and natural killer cells[7]. B cells are involved in humoral immunity as memory B cells and plasma cells that are responsible for generating and secreting antigen specific antibodies against recognized antigens[8]. T cells have several subsets and are involved in stimulating T cell responses to infection, inducing differentiation and maturation of B cells and participating in destruction of virally infected cells[9]. Finally, natural killer cells are important in the innate immune response where they rapidly recognize and destroy compromised cells such as virally infected or cancerous cells[10].

To differentiate into the myeloid lineage, the stem cell becomes a common myeloid progenitor and can terminally differentiate into thrombocytes, erythrocytes, monocytes and granulocytes, which consist of three subtypes, eosinophils, neutrophils and basophils[6]. Thrombocytes are important for the generation of platelets and other factors important in the clotting process[11]. Erythrocytes are colloquially known as red

blood cells which are carriers of oxygen due to the presence of hemoglobin[6]. These cells supply oxygen to the organs and tissues of the body and participate in the removal of waste such as carbon dioxide[6]. Monocytes are phagocytic cells that mature into macrophages which participate in the immune response and removal of damaged cells through phagocytosis[6]. Granulocytes contain distinct secretory vesicles called granules which contain cytotoxic molecules such as degradative enzymes or peptides[12]. Granulocytes respond to infection through a degranulation process, releasing the contents of the granules. Basophils and eosinophils are rare – basophils participate in the inflammatory immune response while eosinophils are important for parasitic infections[12]. Neutrophils are the most predominant granulocytes and are responsible for the destruction of compromised cells through phagocytosis and granule-mediated release of anti-microbial products[12].

1.11 Leukemia

Leukemia is ultimately an interruption in the differentiation process of the blood stem cells in either the lymphoid or myeloid lineage at a precursor stage, resulting in an over proliferation of the precursor cells and the absence of the respective mature cell type[13,14]. This is due to the more apoptosis-resistant and hyper-proliferative phenotype of the leukemic blasts. The resultant high abundance of these non-functional precursor cells in the sites of hematopoietic cell development such as the bone marrow outcompetes the normal cells present and eventually prevents the production of normal and healthy counterparts[13]. This disrupts the normal physiological function of the blood cell types, and depending on the cell type affected, interferes with the ability of the body to control blood coagulation, respond to infections or provide oxygen causing anemia[13].

Leukemia is classified based on lineage of blood cells impacted - lymphocytic leukemia involves the lymphoid cells and myelogenous leukemia involves the myeloid cells[15]. Leukemia can be further classified based on the length of the development of the disease. Acute leukemia develops quickly in a short duration of time where immature lymphocytic or myelogenous cells are proliferating inappropriately[15]. Symptoms manifests quickly due to the excess immature blood cells and subsequently require urgent treatment before the disease becomes severe[15]. In contrast, chronic leukemia develops over a long period of time and involves a gradually increasing accumulation of mature lymphocytic or myelogenous cells that do not function properly[15]. Consequently, chronic leukemia can be asymptomatic for long durations since it develops more slowly and can be overlooked or attributed to other causes[15]. Generally, acute leukemia is more common in children while chronic leukemia develops in older individuals[15].

Based on these classification systems, leukemia can be categorized into acute lymphocytic leukemia, acute myeloid leukemia, chronic lymphocytic leukemia or chronic myeloid leukemia[15]. There are other types of leukemia that are rare and only partially qualify to be classified based on these systems. Hairy cell leukemia is a chronic form of leukemia that involves the excess proliferation of B cells that are abnormal and appear with a distinct "hairy" appearance[15]. Myelodysplastic syndrome is a pre-malignant disease to AML characterized by poorly developed myeloid cells that die prematurely, eventually depleting healthy cells[15].

Due to the diverse characteristics of the leukemia types, treatment options depend on the type of leukemia and range from general chemotherapy with cytotoxic agents or targeted treatments to eradicate leukemic cells to stem cell transplants to replace the malignant blood cells with non-cancerous cells from a donor[15]. Targeted treatment

options are available for some types of leukemia, such as acute promyelocytic leukemia due to their unique characteristics which offer a specific target for therapeutic intervention.

1.12 Acute Promyelocytic Leukemia – a distinct subset of AML

Acute promyelocytic leukemia (APL) is a distinct subtype of acute myeloid leukemia (AML) based on cytogenetic and morphological characteristics and accounts for approximately 10-15% of adult AML cases[13,16,17]. Despite being more common in the older population, APL is not gender or age restricted, although the risk of APL can be elevated in patients with a history of bone marrow disorders or previous chemotherapy exposure[15].

APL is characterized by arrested differentiation in the myeloid lineage of hematopoietic stem cells, specifically at the promyelocyte stage, resulting in the over accumulation of these granulocyte precursors and the absence of mature granulocytes[18]. These immature cells are considered the leukemic blasts due to their hyperproliferative nature and usually appear hypergranular with excessive expression of primary azurophilic granules[19]. Eventually, the promyelocytes outcompete the local cells in the bone marrow which depletes the levels of normal white and red blood cells as well as platelets, disrupting normal blood dynamics and causing the severe symptoms associated with the disease[18]. APL can be stratified based on the severity of the disease – patients considered to be low risk have a white blood cell (WBC) count of 10,000/microliter or less while patients considered high risk have a WBC greater than 10,000/microliter[13].

Historically, APL was associated with high mortality and poor patient outcomes due to the severe bleeding and systemic hemorrhagic symptoms characteristic of the

disease. These symptoms contributed to early patient death before and even during treatment with chemotherapy, making immediate therapeutic intervention essential[20,21]. In a cohort of 116 APL patients, 77.6% of those patients experienced severe bleeding and hemorrhagic events before the onset of treatment[22]. Additionally, hemorrhage-associated death was responsible for the majority of treatment failures in another study involving a cohort of 732 APL patients undergoing treatment with all-trans retinoic acid (ATRA) in combination with the chemotherapeutic agent idarubicin[23].

The risk of hemorrhage is due to dysfunctional blood clotting resulting from the disruption of normal hemostasis in APL, which can manifest into a systemic condition called disseminated intravascular coagulation (DIC). The two hallmarks of DIC is hypercoagulability and hyperfibrinolysis [24]. Hyper coagulation is excessive coagulation activity, which normally controls blood clotting through platelet and fibrin activation, resulting in excess clot formation. In APL patients, many clotting activating proteins such as fibrinopeptide A is significantly elevated and pro-coagulant proteins such as tissue factor (TF) and cancer procoagulant (CP) are aberrantly produced and released by APL cells[25,26]. Hyperfibrinolysis is excessive fibrinolytic activity which degrades fibrin and inhibit platelet aggregation, resulting in compromised blood clotting and the risk of severe bleeding[26]. APL patients have decreased levels of proteins necessary for blood clotting such plasminogen and plasminogen activator inhibitor 1 (PAI-1) and more importantly, have elevated levels of annexin II which is required for the conversion of plasminogen into plasmin, an enzyme necessary for the degradation of blood clots[26]. Together, excessive blood clots blocking the blood vessels of the body combined with impaired platelet and blood clotting activity results in the severe bleeding and hemorrhagic symptoms of DIC.

With the advent of highly curative treatment regimes, the risk of DIC has been minimized and APL has become a mostly survivable and manageable disease with a favorable survival outcome. The use of plasma and platelet transfusions to replenish fibrinogen and platelet levels in combination with ATRA treatment has also achieved partial success in reducing the risk of developing DIC in APL patients[27].

Despite these advancements, APL patients remain susceptible to treatment failure through hemorrhagic death due to the nature of induction and chemotherapy treatment. These treatments exacerbate the risk of hemorrhagic symptoms. ATRA induced differentiation can inadvertently create a hypercoagulable environment due to the restoration of the granulocyte population in differentiation syndrome. The cytotoxic nature of chemotherapeutic agents results in the lysis and release of pro coagulant factors such as microparticles (MP) or CP, as well the release of the enzymatic contents of promyelocytic granules [25,26].

1.13 PML-RARα – a key initiation event

Uniquely, the cause in 98% of APL cases is attributed to a somatic single chromosomal translocation event between chromosome 15 and chromosome 17[28]. The chromosomal translocation is a reciprocal exchange of a region between the two chromosomes[28]. This produces an aberrant fusion between the promyelocytic leukemia (*PML*) at 15q22 and retinoic acid receptor α (*RAR*α) genes at 17q21, resulting in the formation of the PML-RARα fusion protein important for APL pathogenesis[28,29].

Normally, the RARα protein is responsible for regulating transcription and gene expression by responding to its respective ligands, retinoic acid or 9-cis retinoic acid. In the absence of either ligands, RARα associates with the cofactor RAR-retinoid-X-

receptor (RXR), forming a RXR/RAR heterodimer that binds to complementary regions on DNA called retinoic acid response elements (RARE)[30]. The RXR/RAR heterodimer silences the transcription of the gene associated with the RARE site by coupling to co-repressor molecules such as histone deacetylases (HDAC), which promote repressive chromatin interactions with histones. In response to physiological levels of retinoic acid, the RXR/RAR heterodimer dissociates from the co-repressor complex and instead interacts with co-activators, promoting the transcription of the associated genetic material. Conversely, PML functions as a tumor suppressor by regulating cell division and replication as well as apoptosis through interactions with many binding partners such as p53[31]. In response to sources of cellular stress, the PML protein localize and form specialized structures called PML nuclear bodies (PML-NB) through a multimerization process between sumolayted PML proteins as well as other proteins[1]. These PML-NB structures interact with a myriad of proteins and transcription factors such as p53 to regulate apoptosis and important cellular processes[32]. Additionally, the PML-NB structure interacts with chromatin through protein extensions of its own composition which enables the regulation of transcriptional programs[32]. Together, PML-NBs help keep cells function normally.

Although the resultant chimeric PML-RARα protein retains the majority of the domains of the PML and RARα protein, their functions become aberrantly changed[33]. The *RARα* region becomes truncated due to the break point of the fusion event, resulting in the chimeric protein missing the first exon of *RARα* gene but maintaining the regions involved in DNA binding and ligand response[34]. As a result, PML-RARα behaves as an altered RAR nuclear receptor, where the heterodimer it forms with RXR interacts with the RARE sites in a dominant manner in comparison to normal RARA/RXRA mediated

transcriptional regulation, resulting in constitutive silencing[33]. The PML-RARα fusion protein also acquires the ability to homodimerize which changes the DNA binding specificity of the RARα protein – this complex now is capable of binding to atypical RARE sites with which it normally does not interact[35].

Furthermore, the PML-RARα interacts with corepressors in an inappropriately enhanced manner and no longer responds to physiological levels of the retinoid ligands that promote dissociation of the repressive complex, subsequently repressing transcriptional programs in an unprecedented manner[35]. In the context of APL, RARα regulates genes important in granulocytic differentiation as well as self-renewal[30]. Consequently, due to the altered behavior of the PML-RARα fusion protein, the differentiation process is abrogated and immortalization of the promyelocytes is promoted[30]. Additionally, the loss of the PML protein function results in the uncontrolled hyper proliferation of the promyelocytes due to the loss of cell cycle, DNA repair mechanisms and apoptosis control mediated by the PML-NB[36]. Together, this contributes to the initiation of APL disease.

Diagnosis of APL depends on the detection of the *PML-RARα* fusion and was traditionally done using molecular cytogenetic techniques such as fluorescent *in situ* hybridization (FISH) to visualize the fusion or chromosome analysis using karyotyping to detect the genetic rearrangement[16]. The advent of reverse transcriptase polymerase chain reaction (RT-PCR) as a diagnostic tool has enabled the discovery of other fusion events in APL. Although the vast majority of APL cases are characterized by this PML-RARα fusion protein, there are 8 other characterized genetic rearrangements between *RARα* and other genes such as promyelocytic leukemia zinc finger (*PLZF*) or signal transducer and activator of transcription 5b (*STAT5B*) that result in APL (Table 2)[28,37–39]. Significantly,

the present mutation detected can determine the treatment options of APL disease where some fusion proteins are responsive to all-trans retinoic acid while others are not. These patients have a poorer prognosis relative to ATRA-responsive patients[37,40].

Table 2. Other RARα fusion partners in APL.

RARα fusion partner	Chromosomal location	ATRA-sensitive
BCL6 corepressor (*BCOR*)	X	Yes
Factor interacting with PAPOLA and CPSF1 (*FIP1L1*)	4q12	Yes
Interferon Regulatory Factor 2 Binding Protein 2 *(IRF2BP2)*	1q42.	Yes
Nucleic acid binding protein 1(*NABP1*) or Oligonucleotide/oligosaccharide-binding fold containing 2A *(OBFC2A)*	2q32	Yes
Nuclear matrix associated 1 (NUMA1)	11q13.4	Yes
Nucleophosmin (NPM1)	5q35	Yes
Promyelocytic leukemia zinc finger (PLZHF) or Zinc Finger or BTB Domain Containing 16 (*ZBTB16)*	11q23	No
Protein kinase A regulatory subunit 1α (PRKAR1A)	17q24	Yes
Signal transducer and activator of transcription 5b (STAT5B)	17q21.1–21.2	No

1.14 The Epigenetics Paradigm of APL

Although the PML-RARα translocation event is responsible for initiation of APL, it is not the only contributing factor to disease development and progression. The role of epigenetics, superficial modifications to DNA that regulate gene transcription without altering the DNA sequence, plays a central role in APL development and subsequently treatment response. Similar to other malignancies, APL cells are characterized by aberrant epigenetic changes to the DNA methylation and histone modification profiles, resulting in the repression of normal transcriptional programs critical for granulocytic differentiation and tumor suppression, and contributing to blocked granulocyte differentiation and disease progression[41].

DNA methylation is a repressive epigenetic modification involving the addition of a methyl moiety (-CH3), mediated by a family of enzymes called DNA methyl transferases (DMNT), to a cytosine–guanine dinucleotide (CpG) site, creating a 5-methylcytosine (5-mC)[42,43]. A significant portion of the genes in the genome, up to 60%, contain regions within their bodies rich in CpG sites – these clusters are called CpG islands and are usually localized within the promoter region of genes, which are transcription start sites[44]. In the context of transcriptional regulation, the addition of a methyl group results in a prominent protrusion that sterically inhibit the transcriptional machinery from associating with the promoter region, therefore repressing transcription of the downstream gene[44].

Conversely, the reactivation of gene expression requires DNA demethylation, which is the removal of a methyl group mediated by the ten-eleven translocation (TET) family of enzymes[44]. This process involves the transient conversion of the 5-mC into a 5-hydroxymethylcytosine before being converted back into an unmethylated cytosine[44].

Histone modifications are post-translational modifications to the tails of histone proteins that interact with DNA[45,46]. Chromosomes are composed of chromatin, which is formed of nucleosomes, which are octamer complexes containing 2 sets of 4 different classes of histone proteins, H2A, H2B, H3, and H4, in association with DNA[46]. In the context of transcriptional regulation, the strength of the histone interaction with the chromatin is altered depending on the histone modification[46]. Restrictive histone modifications enhance the histone-chromatin interaction, promoting the condensation of chromatin into transcriptionally inactive heterochromatin. Repressive modifications include histone deacetylase (HDAC) mediated removal of acetyl groups (-COCH3) from the lysine residues on histone 3 through a hydrolytic process, specifically at histone 3 (H3) or histone 4 (H4) proteins[46]. The lysine groups on specific histone proteins such as H3 can also methylated by histone methyltransferases (HMT) through the addition of one (mono-), two (di-) or three (tri-) methyl groups, resulting in the formation of heterochromatin, as well as the silencing of gene expression in euchromatin[46]. SUV39H1 is an HMT enzyme responsible for the tri-methylation of lysine 9 on histone 3 (H3K9me3), while the polycomb repressive complex 1 and 2 (PRC1/PRC2) associated with EZH2 mediates the tri-methylation of lysine 27 on histone 3 (H3K27me3)[46,47]. Permissive histone modifications decrease the histone-chromatin interaction, which promotes transcriptional activity. Histone acetyltransferase (HAT) are a class of enzymes that mediate the addition of an acetyl group to the lysine groups of histones such as lysine 9 of histone 3 (H3K9) [46].

Together, the transcriptionally permissive and repressive modifications at the DNA methylation and histone modification level are important in the regulation of many vital cellular processes such as cell cycle progression, DNA repair and DNA replication.

Therefore, aberrant alterations to the pattern of DNA methylation and histone modification can result in the pathogenesis of many diseases, including cancer.

In APL, there are both genome-wide and gene specific epigenetic changes at the DNA methylation and histone modification level due to the interaction of PML-RARα with histone modifying enzymes HDAC, SUV39H1 and PRC1 and PRC2 as well as DNMTs[48].

In the context of histone modifications, PML-RARα binding is associated with overall decreased transcriptionally permissive histone marks (e.g., H3K9/14ac) and increased transcriptionally repressive histone marks (e.g., H3K9me3 or H3K27me3) at these regions, resulting in repressed transcription and heterochromatin formation[49]. In a study using chromatin-immunoprecipitation coupled with deep sequencing (ChIP-seq), 2722 PML-RARα binding sites were characterized in both the APL NB4 cell line and APL patient blasts, which resulted in the identification of PML-RARα binding target regions with deceased histone acetylation and increased tri-methylation such as retinoic acid receptor beta (*RARβ)*, transglutaminase 2 (*TGM2)*, PML-RARA regulated adaptor molecule 1 (*PRAM1)* and runt related transcription factor 1 (*RUNX1)*[49].

Global DNA methylation also plays a role in APL pathogenesis. APL patient samples have increased genome-wide level of DNA methylation in comparison to normal CD34(+) cells collected from healthy donors and bone marrow cells collected from APL patients in remission[42,50]. Additionally, when comparing the methylome (the DNA methylation profile) of APL patient samples in comparison to other subsets of AML and non-AML patient samples, APL patients have a distinct DNA methylation pattern different from the other samples, with distinct regions of hyper methylation[51].

PML-RARα has also been characterized to associate with DNMT3a, which is overexpressed in APL, in an elevated capacity contributing to the progression of APL disease[52]. Gene specific methylation also plays an important role in APL pathogenesis. Specific genes such as *RARβ* or *TGM2* important for granulocyte differentiation are also hypermethylated and silenced in APL[50,53,54].

Although PML-RARα binding to DNA is sufficient to induce histone modification changes, DNA methylation changes can occur independent of PML-RARα binding. In a study which analyzed the DNA methylation of PML-RARα knock-in mice, the collected myeloid cells had no changes in DNA methylation and hematopoietic stem cells induced to express PML-RARα demonstrated arrested differentiation but no changes in DNA methylation[42]. Therefore, DNA methylation changes are believed to be a late event, occurring later in leukemogenesis and is associated with loss of transcription factor binding[42]. Subsequently, the eradication of the PML-RARα fusion protein may not be enough to abolish APL disease and instead, reversing the DNA methylation changes associated with APL may be the key to successful treatment.

1.15 Historical approach to APL treatment

Although APL is currently considered a disease with a favorable prognosis, it was once considered a fatal malignancy. APL was first recognized as a distinct malignancy in the late 50s by several clinicians who observed severe hemorrhagic symptoms in rapidly deteriorating patients presenting with abnormal levels of promyelocytes[21]. The hemorrhagic DIC symptom subsequently became the hallmark of this disease.

Initially, APL was treated with anthracycline-based regimens due to a landmark study done by Jean Bernard, who is credited as one of the first researchers to identify APL[55]. Bernard observed patients treated with the anthracycline agent daunorubicin alone experienced enhanced complete remission (CR) in comparison to treatment using other chemotherapeutic agents such as methotrexate or 6-mercaptopurine[55]. The class of anthracyclines function as topoisomerase II inhibitors which prevent the proliferation of cancerous cells through interfering with DNA replication[56]. Although this treatment was effective in curing approximately 50% of patients treated, APL patients remained susceptible to the hemorrhagic symptoms due to lowered blood cell production caused by danurobicin and subsequently experienced treatment failure[55]. Compounding this complication was the fact that danurobicin had severe side effects such as fatal cardiac toxicity due to its interference with heart function[56]. Despite this, anthracycline-based treatment remained the standard course in APL treatment for decades due to its relatively high level of response. The treatment was slightly improved by supplementing treatment with platelet and plasma transfusions to counter the lowered blood cell production[26].

Although indirect, the advent of molecular cytogenetics played a crucial role in improving the treatment and survival of APL patients by improving disease identification, which decreased the time it took for patients to begin treatment. Early intervention is essential for preventing the fatal symptoms of APL and minimizing the early deaths that patients experienced which was the largest cause of treatment failure[57].

Disease identification was initially dependent on the recognition of certain morphological changes in promyelocytes such as elevated levels of the myelodoperoxidase (MPO) enzymes and or granules observed through staining and microscopy techniques. With the adoption of molecular cytogenetic techniques, the

discovery of the translocation between chromosome 15 and 17 was made by Janet Rowley who observed the presence of this translocation in all the leukemic blast samples studied[58]. The chromosomal translocation was further characterized through repeated cloning of the fused chromosomes, and the fusion between the PML protein and RARα protein was elucidated[28]. The use of RT-PCR and modern FISH to quickly diagnose APL also has a therapeutic component– these rapid techniques enable the monitoring of disease during treatment to determine treatment success and identify potential disease relapse or impending hemorrhagic symptoms.

1.16 Retinoic Acid – advent of induction therapy

Serendipitously, the discovery of using all-trans retinoic acid (ATRA), a ligand for RARα, in the treatment of APL was made in China in 1985 before the knowledge of this chromosomal translocation event was known. In an attempt to minimize the costs associated with chemotherapy treatment, the use of ATRA was assessed in for its potential use in the treatment of APL. Twenty three out of 24 APL patients treated with ATRA achieved complete remission of the disease without the characteristic side effects accompanying chemotherapy such as decreased myeloid cell counts or increased coagulopathy associated with hemorrhagic symptoms[59]. Since this first introduction of ATRA as an induction therapy for APL, it has had a dramatic impact in the survival outcomes of this once deadly disease. Subsequent studies observed the effectiveness of ATRA in inducing complete remission in different cohorts of patients, with minimal side effects associated with chemotherapy such as hypoplasia and low morbidity[60,61]. Further characterization of ATRA treatment in the APL NB4 cell line later identified the mechanism of action through induced differentiation, degradation and restored maturation

of leukemic blasts into granulocytes[62]. ATRA was then combined with anthracycline therapy as the standard front-line treatment option for APL patients.

In the context of treatment, the oncogenic fusion gene provides an ideal target for therapeutic intervention due to the specificity of the ATRA to the RARα moiety of the fusion protein. At supraphysiological levels, ATRA stimulates degradation of the chimeric PML-RARα in APL cells through a ubiquitin-proteasome pathway, subsequently restoring normal RAR transcriptional programs and granulocyte differentiation that was interrupted by the fusion protein [30,48,63]. Additionally, the activation of caspases through PML-RARα degradation also leads to leukemic cell death.

Due to the role of RARα in transcriptional regulation, identifying the downstream transcriptional changes caused by ATRA treatment is important in understanding the molecular mechanism of the treatment. To this effect, microarray experiments have been performed to identify the pattern of gene expression induced by ATRA treatment, which initially identified 100 upregulated and 69 downregulated genes potentially regulated by ATRA involved in a variety of cellular processes such as differentiation and cell cycle control[64]. Several ATRA-induced target genes important in APL pathology have been identified, including CCAAT/enhancer binding protein (*C/EBP*) which is required for neutrophil specific differentiation or *RARβ* which controls granulocytic differentiation programs and is aberrantly hyper methylated and subsequently silenced in APL cells. Other genes have been identified to play a role in other components of APL such as *TGM2*, which in addition to controlling the differentiation of leukemic promyelocytes into mature granulocytes, is also important to restore normal coagulation dynamics and required for response to ATRA[53,54].

Improvements in transcriptome analysis techniques have improved the scale of gene expression analysis to thousands of genes and have interestingly revealed that most of the ATRA-modulated genes are actually distant from locations to which PML-RARα binds[49]. Therefore, the gene expression changes induced by ATRA may be due to changes in other mechanisms of transcriptional control rather than PML-RARα mediated silencing, such as DNA methylation or histone modifications. In terms of its effects on the aberrant epigenome of APL cells, ATRA induces genome-wide transcription activating histone acetylation (e.g., H3K9ac and H3K9/14ac) of target genes, but it has negligible effects on the transcriptionally repressive histone methylation (e.g., H3K9me3 and H3K27me3) and DNA methylation[42,45,48–50]. Therefore, the aberrant DNA methylation characteristic of APL disease remains unaffected after ATRA treatment.

Although ATRA was successful in inducing short-term remission in approximately 80% of patients and was appealing as a non-chemotherapeutic treatment, it could not achieve acceptable long term success in complete remission of patients [18,27,59,60]. Additionally, some patients treated with ATRA alone experienced a unique combination of symptoms such as fever and vascular capillary leakage now called differentiation syndrome, which can escalate to fatal acute renal failure[65]. This is due to excessive restoration of the granulocyte population and inappropriate release of interleukins and inflammatory factors, although a complete understanding remains unknown[65].
Its subsequent combination with anthracycline-based chemotherapies significantly reduced disease relapse and increased response rates to 75%–80% in the treatment of newly diagnosed patients[66]. ATRA served as the induction phase of therapy and the chemotherapeutic agents daunorubicin or idarubicin served as the consolidation phase of therapy. It is important to administer the treatment in sequence to achieve successful

leukemic blast differentiation followed by the cytotoxic effects of the chemotherapeutics. This combination significantly reduced the disease relapse experienced by patients treated with either agent alone and reduced the occurrence of differentiation syndrome. Another treatment complication is the acquired resistance to ATRA that some patients develop, which becomes especially problematic in patients with relapsed disease who cannot respond to subsequent ATRA treatment. Evidence indicates that this acquired resistance is due to mutations at the PML-RARα fusion protein, specifically at the RAR*α* moiety to which ATRA corresponds. Although non-chemotherapeutic treatment initially seemed feasible for APL, its success at this point remained out of reach and ATRA combined with consolidation treatment was considered the first line treatment option.

1.17 Arsenic Trioxide – augmenting induction therapy

Arsenic trioxide (ATO) historically was used in ancient Chinese medicine and its discovery for the use in the treatment of APL was due to a study that assessed the efficacy of ATO to treat a panel of over 1000 cases of cancer. This study identified ATO to have a therapeutic effect specifically in APL patients. Subsequent studies identified significant complete remission rates in newly diagnosed patients, up to an 80% rate, when treated with ATO alone followed by high disease-free survival and overall survival rates[67]. Additionally, ATO treatment did not result in the bleeding symptoms associated with ATRA and chemotherapy treatment due to its negligible effect on other bone marrow cells and had comparable successful for treatment of APL, making it a favorable option. Encouragingly, ATO has been shown to successfully induce remission in patients with relapsed APL, who have a much poorer prognosis, to a comparable level to new APL patients[67,68].

ATO has specificity for the PML moiety of the PML-RARα fusion protein. At low doses (0.5μM), ATO works by inducing degradation of the PML-RARα in leukemic blasts through a proteasome mediated pathway, restoring the formation of PML-NBs. ATO also induces modest granulocytic differentiation of APL cells at low doses, although this mechanism remains poorly understood[18,51,67,69,70] . In higher doses (over 1.0μM), ATO is capable of inducing apoptosis of leukemic blasts through a mitochondria-mediated pathway involving cytochrome c release and activation of caspase-9.

Similar to ATRA, ATO treatment induces transcriptional changes in gene expression, modulating the upregulation and down regulation of hundreds of genes[68]. In terms of its effects on the aberrant epigenome of APL cells, there are reports of the effect of ATO on DNA methylation in APL cells. A recent study showed that 2.0μM ATO reduced DNA methylation and increased mRNA levels of cell cycle related genes in NB4 cell. ATO reduced transcript levels of DNA methyltransferases 1, 3A and 3B in NB4 cells, which would have genome-wide demethylating effects on DNA[71]. This is consistent with another study on the cell line HL-60 (an APL-like cell line that lacks the PML-RARα fusion), in which 1μM ATO modestly reduced global methylation comparable to the DNA-demethylating agent decitabine[72].

Due to its minimal toxicity, ATO presents a potential avenue to treat patients for which chemotherapy is contraindicated, such as older patients who cannot tolerate the side effects associated with chemotherapy. Similar to ATRA treatment, induction treatment with ATO alone also has the risk of the differentiation syndrome due to the induced differentiation caused by the treatment[65]. Therefore, vigilant observations and the use of corticosteroids, such as dexamethasone, is imperative to counteract the risk of this potentially dangerous condition. Due to its success in relapsed patients and relatively low

toxicity, ATO is currently the recommended treatment course for relapsed/refractory patients, especially in patients with ATRA resistance[68].

1.18 Combination Induction Therapy – a chemotherapy-free treatment

Although treatment of APL with the non-chemotherapeutic agents ATRA and ATO has been shown to be successful as in certain contexts, their potential to replace chemotherapy had remained elusive when assessing their effects as single agent treatments. However, there is very convincing evidence in adult patients regarding their synergistic potential when used in combination.

Previous trials have demonstrated that the combination of ATRA with ATO treatment is equivalent to the combination of ATRA and anthracyclines; as a result, treatment recommendations are moving towards this newer non-chemotherapeutic combination[73]. Several large scale clinical studies recently concluded studying this chemotherapy free combination in comparison to treatment regimens with chemotherapy. Together, ATRA and ATO achieve complete remission in 90-100% of patients and overall survival between 87-97% which was comparable to the success rates that ATRA and anthracycline achieved[66,74,75]. Encouragingly, in another cohort of 187 patients composed of 54 high risk and 133 low risk patients, a complete remission rate of 96% was achieved in patients treated with the combination of ATRA and ATO[76]. Following up with the treated subjects, the 5 year disease free and overall survival was 96% and 88%, respectively, indicating that this treatment combination induced long term, durable success[76]. Comparing the combination of ATRA and ATO to treatments using the induction agents individually revealed that although the combination treatment achieved similar rates of complete remission to single agent treatment, the combination treated

patients achieved quicker and longer lasting complete remission, while single agent treated patients experienced high relapse[76].

The mechanisms of the synergy between the two non-chemotherapeutic agents however remains not completely understood. At the molecular level, these two agents have complementary specificity for the PML-RARα fusion protein, where ATO binds to the PML moiety and ATRA binds to the RARα moiety, both promoting degradation of the fusion protein through distinct pathways[73]. An *in vivo* study of the effects of both agents on the leukemic blasts isolated from PML-RARα transgenic mice established that combination ATRA and ATO achieved enhanced granulocyte differentiation and leukemic cell apoptosis, resulting in reduced tumor burden[77]. More significantly, the combination treatment achieved long term remission in mice 9 months after treatment had been terminated, while mice treated with single agent treatment experienced relapse shortly after treatment termination[77]. Therefore, much evidence supports the beneficial outcomes of the treatment, however the need to investigate the mechanism of synergy between these two agents is a valid necessity that has not been achieved yet. Despite evidence regarding the potential complementary effects of ATRA and ATO at the histone modifications and DNA methylation level respectively, the role of epigenetics changes following combination treatment has not been investigated yet.

1.19 Techniques to study epigenetic changes

The key to understanding combination treatment in APL may be at the epigenetic level, due to the involvement of global and gene specific DNA methylation and histone modification changes. Both drugs have been shown to affect the epigenome in some capacity, therefore the changes that they induce may be crucial in understanding how the

synergy between both agents works. There are many tools currently available to study the epigenome.

To study global DNA methylation changes, all the DNA methylation sites needs to be mapped to determine the specific DNA methylation pattern and the changes induced by treatment. The MethylationEPIC BeadChip (Infinium) microarray assesses over 850,000 CpG methylation sites within the human genome, enabling a complete characterization of hypermethylated and hypomethylated regions[78]. Reduced representation bisulfite sequencing (RRBS) is more limited than the 850K assay where it assesses only around 10-15% of all CpGs in genome, however it can assess single base methylation of CpG sites within CpG islands, enabling the characterization of specific methylation patterns at the gene level[79].

Although these techniques enable high resolution characterization of DNA methylation patterns, they are expensive and take time to perform since they are processed off site. There are global methylation assays that quantify the total global DNA methylation levels, which provide a snapshot of the overall change in DNA methylation. These assays are ELISA based and quick to perform, however are inaccurate in assessing DNA methylation levels in inherently low methylated cancer cells[80]. An alternative method to assess global DNA methylation is to use a representative surrogate - long interspersed nucleotide element 1 (LINE-1) is an accepted surrogate for general global methylation levels of the genome[81]. These LINE-1 elements are transposable repetitive elements making up 17% of genomic DNA and are often heavily methylated, holding up to a third of the methylation in the genome[82]. Therefore, measuring the levels of LINE-1 elements in bisulfite-converted DNA can offer an estimate of the level of global DNA methylation in a sample.

Global histone modifications can be studied using chromatin-immunoprecipitation coupled with next generation sequencing technology to isolate the histone protein of interest and characterize all the DNA associated with it[83]. Without the sequencing aspect, ChIP can also be used to interrogate the levels of specific genes associated with the histone of interest. Assessing global levels of histones using histone modification specific antibodies coupled with western blotting offers the total changes in the levels of the histone modifications in the genome.

1.20 APL models of study

The models available to study APL disease remains limited due to the uncommon nature of the disease. Patient samples acquired from the bone marrow or peripheral blood of APL patients present the most ideal option for studying APL disease since it offers a direct representation of the disease in the patient. Patient samples however are difficult to obtain since procuring samples from bone marrow is highly invasive involving biopsy or aspiration techniques. Establishing primary cell lines *ex vivo* is extremely difficult and requires extensive maintenance with cytokines such as granulocyte-macrophage stimulating factor (GM-CSF) and other growth factors to sustain the cells[84]. Additionally, the AML-type cells isolated from the patient sample are quite heterogeneous where the leukemic progenitor cells make up only a small percentage of the collected samples, therefore a lot of effort is required to isolate those specific cells. Finally, there is difficulty in expanding the population of leukemic cells to a favorable number due to the lowered level of telomerase activity associated with AML cells in particular, which are necessary to maintain telomere length[85]. Telomere becomes shortened during cell proliferation, therefore the reduced telomerase activity results in catastrophic loss of telomere base

pairs, resulting in cell death[85]. Ultimately, establishing the population of primary cells necessary to perform the epigenetic and differentiation studies necessary is difficult.

The bona fide cell line available for *in vitro* study is the NB4 cell line, a maturation inducible cell line isolated and immortalized from a 20 year old female patient in 1986[86]. This cell line harbors the t(15:17) translocation and demonstrates a promyelocyte phenotype. Importantly this cell line also demonstrates responsiveness to retinoic acid and becomes differentiated with elevated expression of the CD11b myeloid marker[86]. Although other APL cell lines exist, they all have caveats to their use as a representative model of APL. HL-60 is considered a promyelocyte cell line with granule activity however does not harbor the t(15:17) translocation characteristic of APL[84]. Additionally, it is only semi-responsive to retinoic acid and demonstrates different gene expression profiles in comparison to NB4 and patient cell lines. Microarray analysis between the NB4 and HL-60 cell lines determined both cell lines are non-complementary in their upregulated and downregulated genes under ATRA treatment, making comparison of gene expression between the two cell lines impossible[87]. The PL-21 cell line is considered a pseudo-APL cell line due to its monocytic presentation, despite being derived from an APL patient[84]. Finally, the U937/Pr9 cell line is a monoblastic cell line that has been modified to artificially express the t(15:17) translocation through a zinc inducible promoter which results in acquired APL features such as arrested differentiation and response to retinoic acid[88]. Although this cell line demonstrates similar levels of the PML-RARα fusion protein in comparison to patient samples, the PML-RARα fusion protein does not account for all the changes in APL, especially at the DNA methylation level. Inducing the expression of the fusion protein does not recreate the altered DNA methylation pattern seen in APL patients[42]. Therefore, the use of a cell line dependent on

expressing the PML-RARα protein for studying epigenetic changes would not be completely representative of the changes seen in an APL patient. Ultimately, the NB4 cell line remains the only feasible APL cell line for scope of this study.

A transgenic animal model of APL has been generated that fully recapitulates the features of the disease including arrested granulocyte differentiation and responsiveness to ATRA treatment. This model was created through the introduction of human PML-RARA cDNA into an expression cassette containing a promoter for expression of myeloid cells, which was inserted into the embryos of FVB/N mice[77]. The bone marrow of the generated transgenic mice were confirmed to have robust expression of PML-RARα and interrupted granulocyte differentiation and the mice eventually developed APL disease[77]. This animal model offers important potential for studying the effects of combination treatment in APL in an *in vivo* setting.

1.21 Rationale and Hypothesis

The underlying mechanism behind the reduced relapse rates of the combination treatment is only partly understood and the effects of the combination treatment on epigenetic modifications have not been explored. Evidence indicates that ATRA and ATO alone are capable of exerting effects on histone modifications and DNA methylation, respectively, therefore the complementarity in their effects could be at the epigenetic level. In this study, I aim to compare the short-term, long-term and the post treatment termination effects of ATRA and ATO on NB4 APL cells, and characterize the epigenetic modifications at the global level, specifically the DNA methylation and histone modification changes to identify combination treatment exclusive changes. Therefore, I hypothesize that the combination treatment will induce enhanced and lasting

differentiation of the APL cells to a degree not seen in single agent treated cells which will coincide with changes in DNA methylation and histone modification changes.

Since epigenetic changes are associated with transcription changes, we also want to interrogate a number of genes important in APL disease to determine if differential expression and epigenetic regulation is induced by combination treatment in comparison to single agent treatment. Therefore, I hypothesize the combination treated APL cells will have differential expression of genes associated with changes in DNA methylation and histone modifications.

Together, this data will provide new evidence of the benefits of ATRA and ATO post treatment termination and provide possible underlying epigenetic mechanisms for the reduced relapse associated with the combination treatment.

CHAPTER 2 MATERIALS AND METHODS

2.10 Cell line and culture conditions

NB4 cells (the sole APL cell line expressing the PML-RARα fusion) were obtained from the American Type Culture Collection (ATCC) and cultured in suspension in cell culture grade flasks at 37°C in a humidified atmosphere of 5% CO2 using RPMI-1640 (Invitrogen, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) and antibiotic-antimycotic (Invitrogen, Thermo Fisher Scientific) solution. All-trans retinoic acid (ATRA, Sigma-Aldrich) was dissolved in DMSO as a 100mM stock solution and arsenic trioxide (ATO, Sigma-Aldrich) was dissolved in NaOH as a 100mM stock solution and stored in aliquots at -80^{o}C. Prior to use, they were serially diluted in media to working concentrations.

2.11 Extended treatment protocol

NB4 cells were seeded in RPMI-1640 medium into flasks at approximately 50% confluency under twelve different treatment combinations (Table 3) for 24 hours. 10 x 10^4 cells were then collected from each treatment flask for flow cytometry analysis (Figure 1). The remainder of the cells were then passaged, and the medium was refreshed with their respective treatment conditions to extend treatment for up to 72h and 168h, and 10 x 10^4 cells were collected for flow cytometry analysis for each time point (Figure 1).

2.12 Treatment termination protocol

Alternatively, for the 96h post treatment termination time point, the cells from their respective flask were collected at 72h after treatment initiation under 4 treatment

conditions: no treatment, 1.0μM ATRA alone, 0.5μM ATO alone and combination 1.0μM ATRA and 0.5μM ATO, by centrifugation at 500 x g for 5 min, washed with phosphate buffered saline (PBS) twice to remove residual drug(s) and returned to culture with drug-free treatment medium for another 96h, before termination of the experiment and collection of cells (Figure 3). This treatment termination protocol will be used for the remainder of the experiments after 2.13 Flow cytometry.

2.13 Flow cytometry

Treated NB4 cells were collected at 4 time points for flow cytometry analysis (24h, 72h, 168h and 96h post treatment for 72h). Cells were collected by centrifugation at 500 x g for 5 min and each sample was then washed in PBS and suspended in blocking buffer consisting of PBS supplemented with 1 % FBS and 1% ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich) for 10 min at room temperature and then incubated with 3 μL Alexafluor 488 conjugated anti-human CD11b monoclonal mouse antibody (clone M1/70.15, Invitrogen, Thermo Fisher Scientific) in 100 μL of PBS containing 1% FBS, 1% EDTA for 30 min at 4°C. Afterwards, the cells were centrifuged at 500 x g and resuspended in PBS containing 1% FBS, 1% EDTA and 7-aminoactinomycin D (7-ADD, Biolegend) diluted to 1/50 for 15 min at room temperature. A fluorescence activated cell sorter (FACS) Calibur (BD Pharmingen) was then used to detect the percentage of differentiated cells (CD11b+ only or CD11b+ and 7-AAD+) and dead undifferentiated cells (7-AAD only). The flow cytometry data was analyzed using FCS Express 6 Research Edition software (De Novo Software).

2.14 Quantitative PCR

Total RNA was extracted from treated NB4 cells at two time points (72h and at 96h post treatment termination) using Trizol (Invitrogen, Thermo Fisher Scientific) and a Purelink RNA purification kit (Invitrogen, Thermo Fisher Scientific), following the manufacturer's instructions, with the added step of an on-column DNase I (Invitrogen) treatment for enzymatic DNase elimination. Equal amounts of RNA (0.25µg) was converted into cDNA using an iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's recommendations. Quantitative PCR (qPCR) was performed by using SsoFast EvaGreen Supermix (Bio-Rad) and gene-specific primers (Table 4) with a CFX384 thermocycler touch real-time PCR detection system (Bio-Rad). Standard curves were generated for each primer set on serially-diluted pooled cDNA, and primer efficiencies were incorporated into the CFX Manager software (Bio-Rad). The mRNA levels of each sample were then calculated relative to two reference genes (HRPT1 and TBP) and normalized to their respective no treatment controls.

2.15 Chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR)

At 72h and 96h post treatment termination timepoints, 5 million NB4 cells were collected from each treatment condition at each time point and fixed with 1% formaldehyde for 8 mins and cell fixation was quenched with 1.25M glycine and washed with ice cold PBS. The resulting crosslinked protein–DNA complexes were then lysed with three consecutive lysis buffers 1-3 (Table 5) supplemented with a protease inhibitor cocktail for 15 minutes on ice, followed by 10 min centrifugation at 1500 x g at 4 degree. Each collected pellet was then resuspended in 200 uL of sonication buffer (Table 5) in

polystyrene tubes and sonicated using a Q800R2 sonicator (QSonica) with the following conditions; temperature = 4°C, amplitude = 70%, ON time = 30 mins; cycles = 15S ON, 45S OFF. To confirm sonication resulted in 150-250 bp length fragments, a QIAxcel Advanced System Bioanalyzer was used to analyze the distribution of fragment size. The sonicated samples were then spun at 16000 x g for 10 min to separate cell debris and the collected supernatant was then resuspended in 4 mL of dilution buffer (Table 5). From each sonicated sample, 200 uL was stored as the total input and 500 uL is used for each immunoprecipitation (IP) using ChIP-grade antibodies (Diagenode) directed against IgG H3K9/14ac, H3K9me3 and H3K27me3 (Table 6). The samples were incubated with the antibodies overnight at 4 degree on a nutating mixer at low rotating speed. The antibody tagged DNA-protein complexes from each sample were then precipitated using 15 uL of protein A conjugated Dynabeads (Invitrogen, Thermo Fisher Scientific) and a magnetic rack, then washed five times with wash buffer 1, 2 and 3 (Table 5) to degrade the dynabeads and with TE buffer to remove residual chemicals. To decrosslink the IP-isolated DNA, each sample was washed with elution buffer (Table 5) supplemented with proteinase K at 55 degrees for 30 mins and then at 65 degrees for 8 hours. The DNA from each sample was then isolated using a Purelink PCR purification kit (Invitrogen, Thermo Fisher Scientific) along with their respective total input controls (pre-immunoprecipitation DNA). QPCR analysis was then performed using primers targeting the promoter regions (700 bp before promotor start site) of each gene (Table 4) with equipment and reagents described earlier. Results are expressed as the fold change of the enrichment of the DNA detected under the treatment conditions against the DNA detected under the no treatment conditions. This was determined by dividing the signals obtained

from the ChIP by the signals obtained from the total input control sample and normalizing for the DNA detected by the non-immune IgG (negative control).

2.16 Western Blotting

At 72h and 96h post treatment termination timepoints, 1 million NB4 cells were collected from each treatment condition at each time point, washed with ice cold PBS and resuspended in 100 uL of ice-cold RIPA buffer supplemented with PIC and phosphatase inhibitors (Sigma). The protein lysates were then quantified using a BCA Protein assay kit (Thermo Scientific) and a Spectramax M2 plate reader. Lysates were boiled in Laemmli buffer at 95°C for 5 min, and then 20ug from each sample was loaded into the wells of a precast 4-15% Mini-PROTEAN TGX Stain-Free gel (BioRad) alongside a Precision Plus Protein All Blue Prestained Protein Standard ladder and run at 100V for 1 hour. As a loading control, total protein load was confirmed using the stain-free UV fluorescence feature of the PROTEAN TGX gels prior to protein transfer. The gels were then transferred to a PVDF membrane using a TurboBlot system (BioRad) and run for 7 min. As a blocking step, the membranes were incubated in a blocking solution containing 5% bovine serum albumin (BSA) in pH 7.6 TTBS (20 mM Tris-HCl, 200 mM NaCl, 0.05% Tween-20) for 2 hours at 4 degrees. The membranes were then probed by using primary antibodies anti-rabbit H3K9ac (Table 6), H3K9me3(Table 6) and H3K27me3 (Table 6) diluted in 5% BSA in pH 7.6 TTBS overnight at 4 degrees. Secondary species-specific horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1/5000) were then added to the membranes for 1 hour at room temperature. To detect protein levels present, the membranes were washed with ECL clarity substrate (BioRad) and imaged on

a ChemiDoc imager (BioRad) and then quantified using Image Lab 6.0 software (BioRad).

2.17 Bisulfite Pyrosequencing

Genomic DNA was extracted from treated NB4 cells at 72h and 96h post treatment termination timepoints using the Purelink Genomic DNA mini kit (Invitrogen Thermo Fisher Scientific). DNA purity ($A_{280/260}$) and concentration (ng/ul) was evaluated using 2µl nanodrop with the Take3 micro-volume plate on an Epoch Microplate Spectrophotometer (Biotek, Winooski, VT., USA). Based on previous published methods, 20 ng of template gDNA from each sample was bisulfite-converted using the EpiTect Fast DNA Bisulfite Kit (Cat#: 59824, Qiagen N.V, Venlo, The Netherlands), to convert unmethylated cytosine residues to uracil residues in single-stranded DNA while methylated cytosines remained unmodified. Three custom assays covering the *LINE-1*, *RARβ* and *TGM2* promoters were designed using the PyroMark Assay Design software (v2.0; Qiagen N.V, Venlo, The Netherlands) and validated to amplify single PCR products (*LINE-1* = 400nt, *RARβ* = 400nt, *TGM2* = 428nt) using the PyroMark PCR Kit (Cat#: 978703, Qiagen N.V, Venlo, Netherlands) with the primer sequences listed in Table 7 (Integrated DNA Technologies, Inc. (IDT), Coralville, Iowa, USA). PCR conditions for both assays were as follows: 95°C, 15 minutes; (94°C, 30 sec; 56°C, 30 sec; 72°C, 30 sec) × 50 cycles; and 72°C, 10 min, during which the 5-methylcytosines are amplified as cytosines and the uracils are amplified as thymines. The PCR products were then purified using the QIAquick PCR Purification Kit (Cat#: 28106, Qiagen N.V, Venlo, Netherlands), to remove any residue of the PCR reaction that might interfere with the outcome of the sequencing results. Analysis of gene promoter-specific DNA-methylation

was performed using the PyroMark Q24 Advanced pyrosequencer (Qiagen N.V, Venlo, Netherlands). Each kit protocol was followed according to the manufacture's guidelines.

2.18 Global DNA methylation quantification using Epigentek 5-mC MethylFlash Kit

Using a Purelink Genomic DNA mini kit (Invitrogen Thermo Fisher Scientific) and following the manufacturer's instructions, genomic DNA was extracted from treated NB4 cells at 72h and 96h post treatment termination time points and quantified using a Spectramax M2 platereader to collect 100 ng of gDNA from each sample. Global DNA methylation quantification was then performed using the MethylFlash 5-mC Methylated DNA Quantification ELISA kit (Epigentek Group Inc., Farmingdale, NY) according to the manufacturer's instructions. A standard curve was generated from the optical density detected at 450 nm (OD450), measured with the Spectramax M2 platereader, of stable quantified standard DNA samples in increasing concentrations and the OD450 of the NB4 cells was compared to the standard curve to determine the level of DNA methylation.

2.19 Statistical Analysis

All statistical analyses were performed using GraphPad Prism Version 7. ANOVA (one-way analysis of variance or repeated measures) was performed followed by post-tests Dunnett or Bonferroni (specified in the figure legends), when multiple comparisons were made. Significant p values are represented as follows: * <0.05.

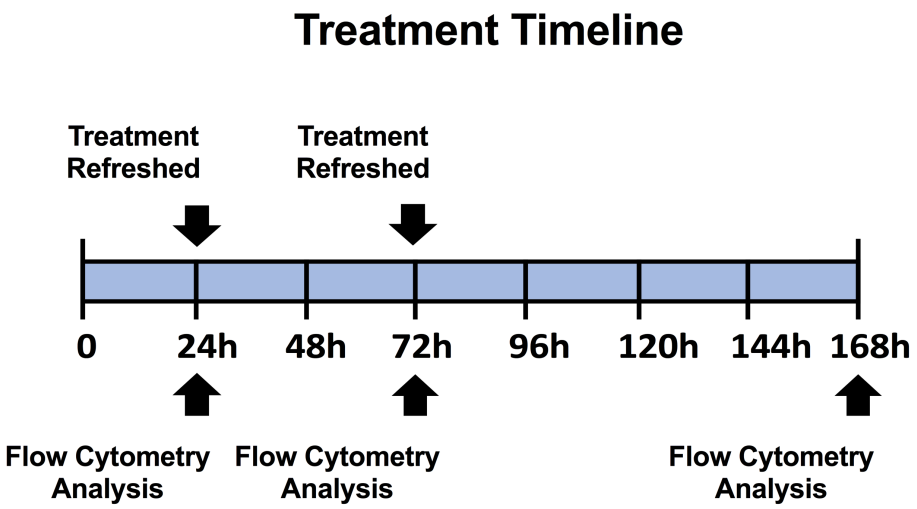


Figure 1. Schematic of extended treatment timeline and the time points NB4 cells were collected for flow cytometry analysis. 10 x 10^4 cells were collected from each treatment condition (Table 3) at the time points indicated.

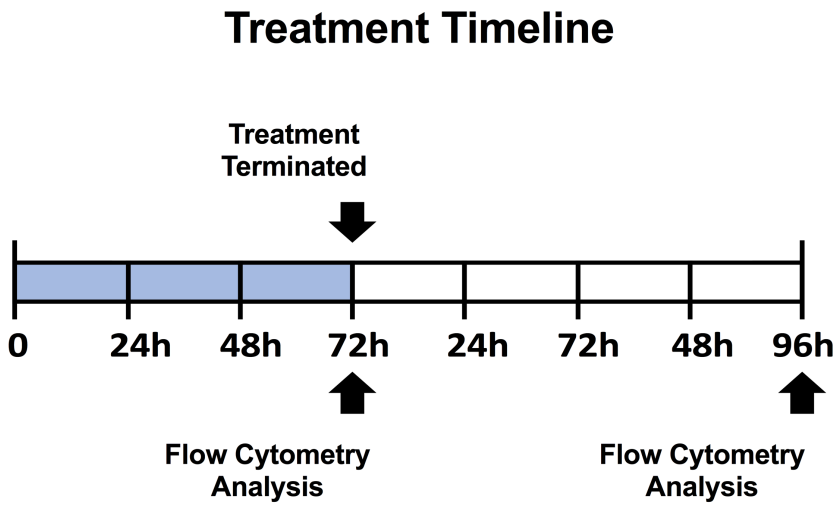


Figure 2. Schematic of treatment termination timeline and the time points NB4 cells were collected for flow cytometry analysis. 10 x 10^4 cells were collected from four treatment conditions: no treatment, 1.0µM ATRA alone, 0.5µM ATO alone and combination 1.0µM ATRA and 0.5µM ATO, at the time points indicated. Treatment was terminated after 72 hours and the remaining cells were resuspended in treatment free media until 96 hours had elapsed.

Table 3. List of drug concentrations used for flow cytometry analysis (selected concentrations for further study bolded)

Concentration	Treatment type
No treatment	Control
0.01μM ATRA	Single agent treatment
0.1μM ATRA	Single agent treatment
1μM ATRA	**Single agent treatment**
0.05μM ATO	Single agent treatment
0.5μM ATO	**Single agent treatment**
0.01μM ATRA + 0.05 μM ATO	Combination treatment
0.01μM ATRA + 0.5 μM ATO	Combination treatment
0.1μM ATRA + 0.05 μM ATO	Combination treatment
0.1μM ATRA + 0.5 μM ATO	Combination treatment
1μM ATRA + 0.05 μM ATO	Combination treatment
1μM ATRA + 0.5 μM ATO	**Combination treatment**

Table 4. Primer sequences for qPCR and ChIP-qPCR analysis

qPCR Primers		
Gene	Forward Primer (5'→3')	Reverse Primer (3'→5')
TGM2	GAGCGAAGGGACGTACTGC	GACAAAGGGCGCATCGTACTT
RARβ	TTCTCAGACGGCCTTACCCT	GCTGGTTGGCAAAGGTGAAC
CCL2	GAAAGTCTCTGCCGCCCTT	GGGGCATTGATTGCATCTGG
ASB2	GAGCCGGACATCTCCAACAA	CACCAGAATCTTCACGGCCT
IER3	CAGAGGACGCCCCTAACG	TGTTGCTGGAGGAAAGTGCT
PRTN3	TGCCGGCCACATAACATTTG	CCCCAGATCACGAAGGAGTC
RPL7a	CAAAAGAGACCTCACCCGCT	CAAAAGAGACCTCACCCGCT
RARα	GTGTCACCGGGACAAGAACT	CGTCAGCGTGTAGCTCTCAG
RAB33A	GCTGGTTGGCAAAGGTGAAC	GCTGGTTGGCAAAGGTGAAC
NDUFB10	CTACTACCACCGGCAGTACC	TCTTCCACTGCATTTCGGCT
NCL	GCTGGTTGGCAAAGGTGAAC	GCTGGTTGGCAAAGGTGAAC
MPO	CGCCAACGTCTTCACCAATG	CATGGGCTGGTACCGATTGT
HIST1H2BK	ACCTCCAGGGAGATCCAGAC	TGTACTTGGTGACGGCCTTG
TBP	GGCAACCACTCCACTGTATCC	GCTGCGGTACAAATCCCAGAA
HPRT1	GACCAGTCAACAGGGGACAT	CCTGACCAAGGAAAGCAAAG
ChIP-qPCR Primers		
Gene	Forward Primer (5'-3')	Reverse Primer (3'-5')
TGM2	CTTCACCGAGCCTCAGTTTC	GATAAGCCCCAGAGGTCACA
RARβ	GGGAGAGAAGTTGGTGCTCAA	CACAAGCCGGCGTTTTCTTT

Table 5. Composition of ChIP reagents

Reagent	Composition
Lysis buffer 1	50 mM HEPES (pH 8), 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Igepal C-630, 0.25% Triton X-100
Lysis buffer 2	10 mM Tris-HCl (pH 8), 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA
Lysis buffer 3	50 mM Tris-HCl (pH 8), 5 mM EDTA, 0.2% SDS
Dilution buffer	2 mM EDTA, 150 mM NaCl, 20 mM Tris-Hcl, (pH 8)
Elution buffer	1% SDS, 0.75% sodium bicarbonate
Wash buffer 1	0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8), 150 mM NaCl
Wash buffer 2	0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris-HCl, (pH 8), 500 mM NaCl
Wash buffer 3	0.25 M LiCl, 1% Igepal C-630, 1 mM EDTA, 10 mM Tris-HC (pH 8), 1% deoxycholate

Table 6. List of primary antibodies for ChIP and western blotting

ChIP Antibodies	**Working concentration**	**Antibody details**
Anti-IgG	1.5ug	Rabbit polyclonal, Diagenode, C15410206
Anti-H3K9/14ac	1.5ug	Rabbit polyclonal, Diagenode, C15410200-50
Anti-H3K9me3	1.5ug	Rabbit polyclonal, Diagenode, C15410193-50
Anti-H3K27me3	1.5ug	Rabbit polyclonal, Diagenode, C15410195-50
Westerns Antibodies	**Working dilution**	**Antibody details**
Anti-H3K9ac	1/5000	Mouse polyclonal, Cell Signaling Technology, C5B11
Anti-H3K9me3	1/2000	Mouse polyclonal, Cell Signaling Technology, 6F12
Anti-H3K27me3	1/2000	Mouse polyclonal, Cell Signaling Technology, C36B11

Table 7. Primers for bisulfite pyro-sequencing

Gene	Forward Primer (5'→3')	Reverse Primer (3'→5')	Sequencing Primers
TGM2	TTGGTGTTTTTTTTTTT TGTTGATGAG	(Biotin)TCTCCTCCTCCC TAAACAAAAT	TTGTTGATGAGG TGG
RARβ	GTTAAAGGGGGGATT AGAATTT	(Biotin)AACTCTACCCC TTTTTTAACA	GGGGGATTAGA ATTTTTTTAT
LINE-1	AGGGAGAGTTAGATA GTG	(Biotin)AACTATAATAA ACTCCACCC	GGAGAGTTAGAT AGTGG

CHAPTER 3 RESULTS

3.10 Flow cytometry analysis of ATRA and ATO treated NB4 cells identifies the treatment combination of ATRA and ATO that achieves enhanced differentiation and cell death

ATRA and ATO have been demonstrated to act in a synergistic manner in the clinic; however, the underlying mechanism has not been fully elucidated. To study this synergistic effect *in vitro*, we first needed to establish the concentration of combination ATRA and ATO that induces more differentiation of APL NB4 cells in comparison to single agent treatment. In the literature, 1.0μM ATRA induces differentiation of NB4 cells without affecting DNA methylation while 0.5μM ATO induces partial differentiation – therefore we investigated these concentrations individually and in combination[89,90]. We also assessed lower concentrations to determine the lowest concentrations of the drugs that would induce differentiation when the drugs were applied together as opposed to singly. We assessed the percentage of differentiated cells (presence of myeloid maturation marker CD11b) and dead cells (7-AAD staining) under these treatment conditions, listed in Table 3, at 24h (Figure 3). After 24h, there was no significant difference in the differentiated CD11b positive cells induced by any of the concentrations of single ATRA and ATO treated cells in comparison to the combinations of treatment. When the treatments were extended to 72h (Figure 4), the combination of 1.0μM ATRA and 0.5μM ATO induced a larger the population of differentiated (CD11b+) cells and that are both CD11b and 7-ADD positive (differentiated cells that had died) in comparison to single ATRA and ATO treatment (Figure 5). The lower concentrations were less effective. Finally, when the treatments were extended to 168h (Figure 6), the population of differentiated cells that were CD11b and 7-AAD positive

became most significantly pronounced under the combination of 1.0μM ATRA and 0.5μM ATO (Figure 7). Additionally, after 168h of treatment the benefits of the combination treatment also became evident even at lower concentrations (e.g. 0.01 μM ATRA + 0.5 μM ATO versus either agent alone at the concentration, Figure 7).

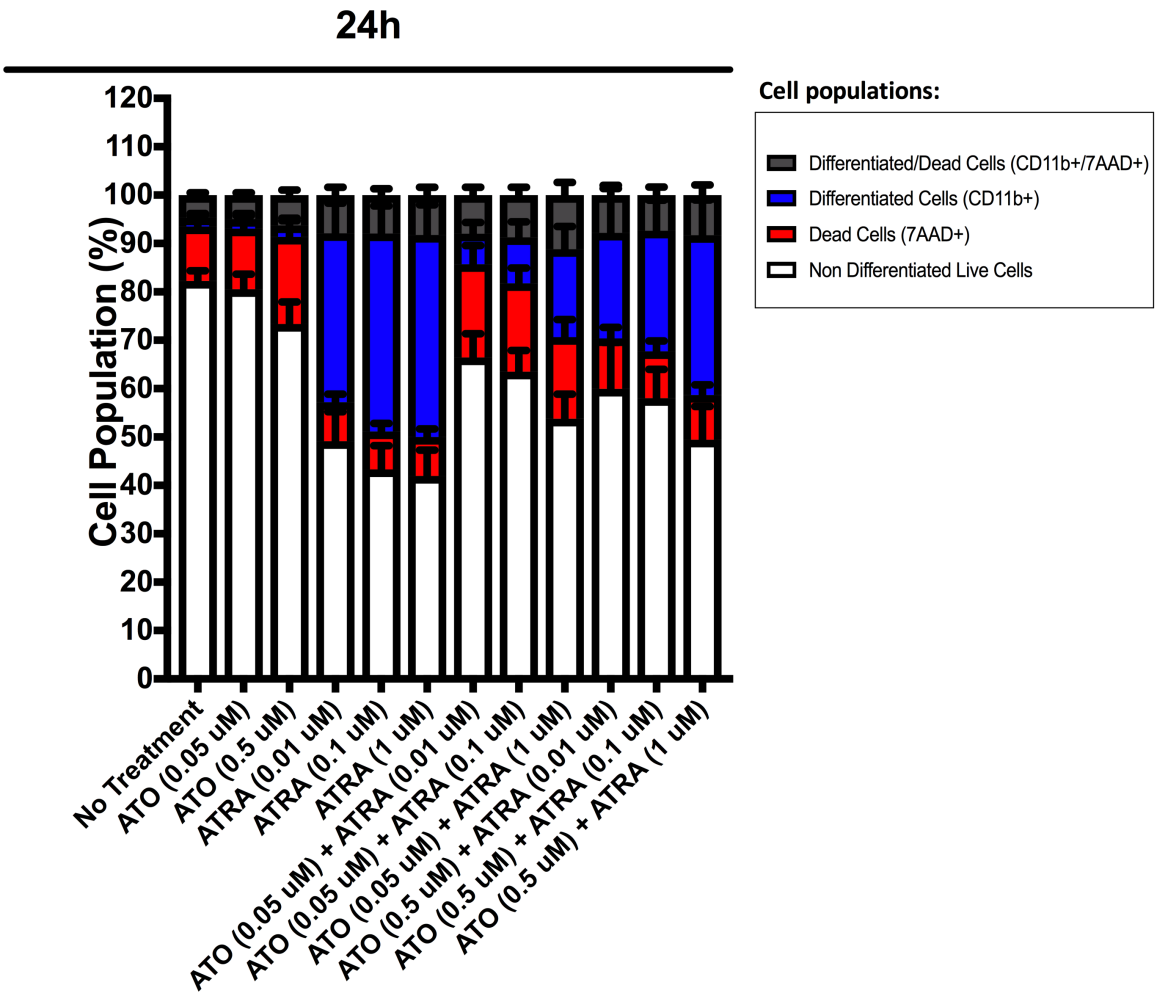


Figure 3. Distribution of differentiated CD11b+ and CD11b+/7-AAD positive NB4 cells under increasing concentrations of ATRA and ATO alone and in combination after 24 hours. Stacked bar graphs summarize the flow cytometry analysis of percentage of dead (7-AAD+) and differentiated (CD11b+ or CD11b+/7-AAD+) NB4 cells induced upon single or combination ATRA and ATO treatment after 24 hours (n=4, error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons).

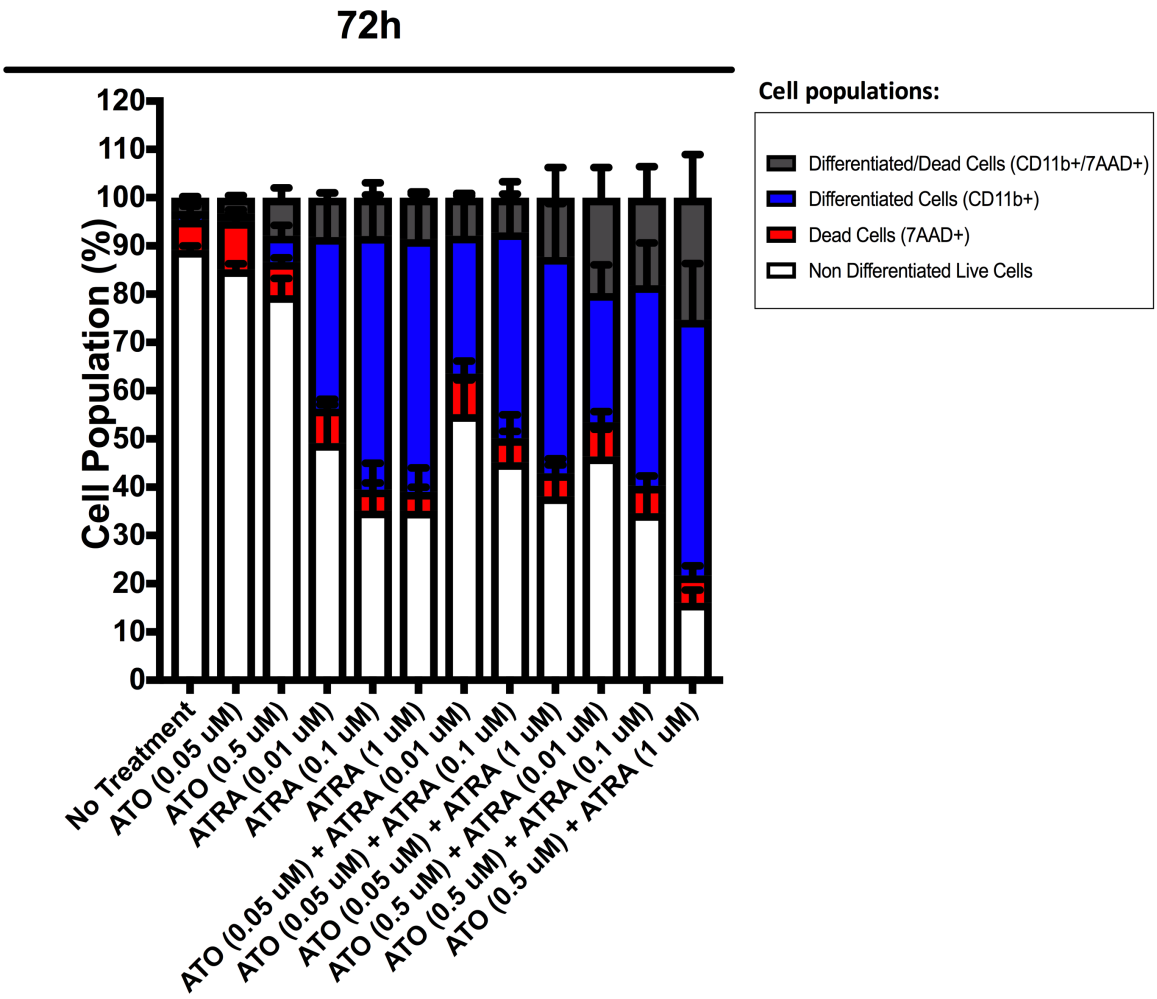


Figure 4. Distribution of differentiated CD11b+ and CD11b+/7-AAD positive NB4 cells under increasing concentrations of ATRA and ATO alone and in combination after 72 hours Stacked bar graphs summarize the flow cytometry analysis of percentage of dead (7-AAD+) and differentiated (CD11b+ or CD11b+/7-AAD+) NB4 cells induced upon single or combination ATRA and ATO treatment after 72 hours (n=4, error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons).

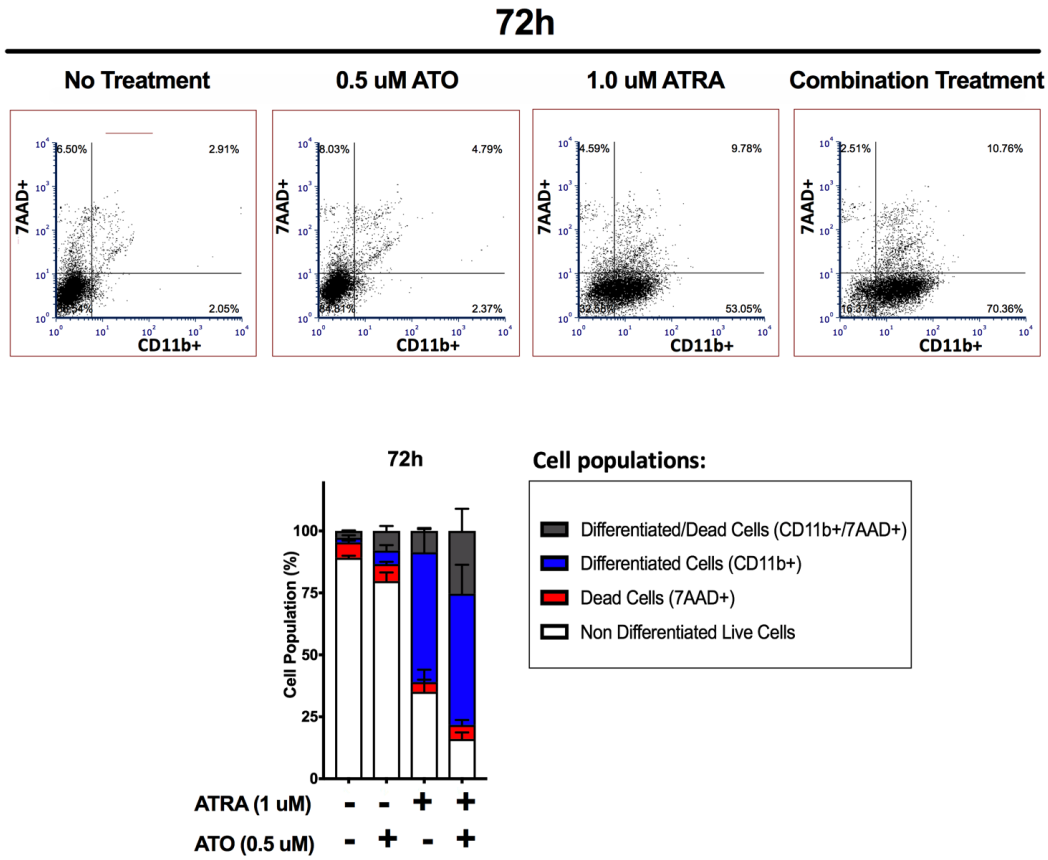


Figure 5. 1.0µM ATRA and 0.5µM ATO induce significant differentiation of APL NB4 cells after 72 hours of treatment. Representative flow cytometry dot plots for CD11b+, 7-AAD+, and CD11b+/7-AAD+ NB4 cells under no treatment, 0.5µM ATO, 1µM ATRA, or combination treatment conditions at 72h. Stacked bar graphs summarize the flow cytometry analysis of percentage dead (7-AAD+) and differentiated (CD11b+ or CD11b+/7-AAD+) NB4 cells induced by 0.5µM ATO, 1µM ATRA, or combination treatment after 72h of continuous treatment (n=4, error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons).

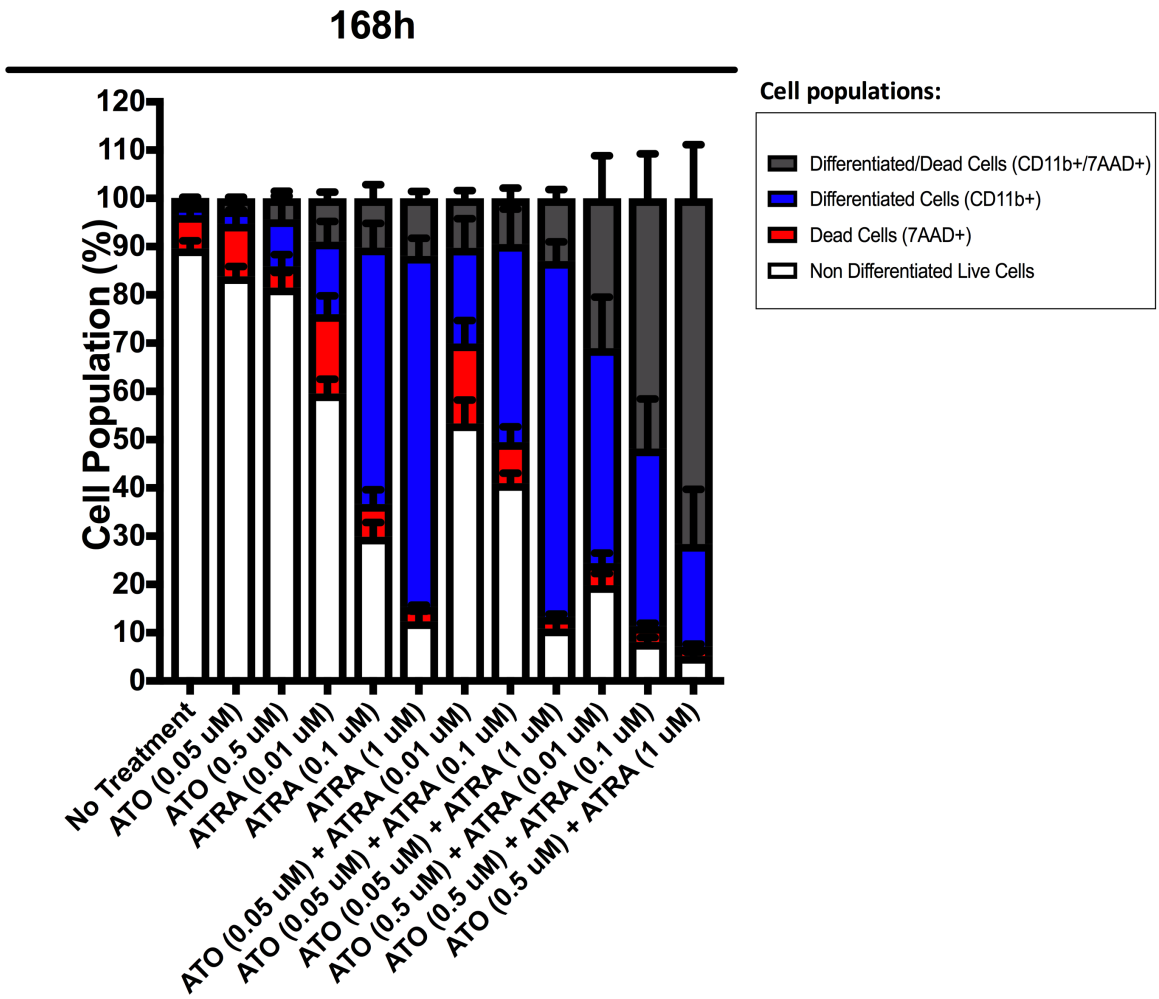


Figure 6. Distribution of differentiated CD11b+ and CD11b+/7-AAD positive NB4 cells under increasing concentrations of ATRA and ATO alone and in combination after 168 hours Stacked bar graphs summarize the flow cytometry analysis of percentage of dead (7-AAD+) and differentiated (CD11b+ or CD11b+/7-AAD+) NB4 cells induced upon single or combination ATRA and ATO treatment after 168 hours (n=4, error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons).

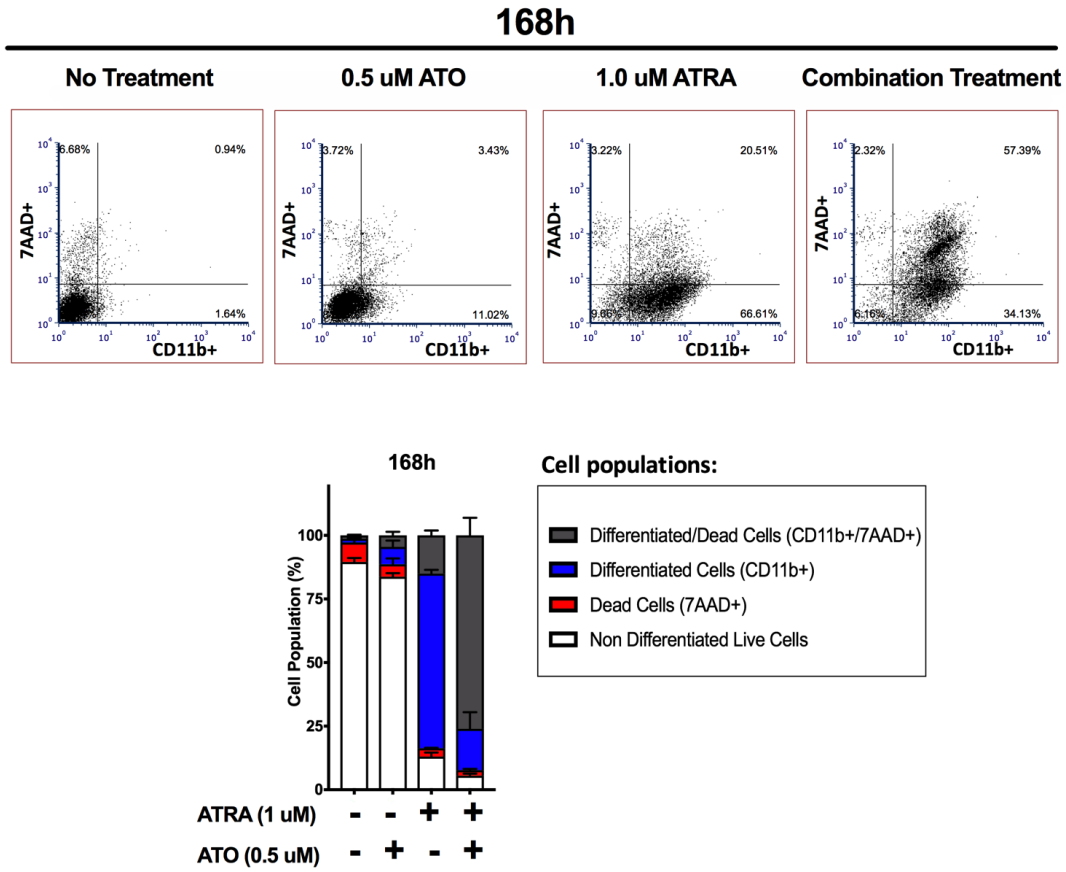


Figure 7. 1.0µM ATRA and 0.5µM ATO induce significantly increased differentiation of APL NB4 cells in comparison to single agent treatment after 168 hours of treatment. Representative flow cytometry dot plots for CD11b+, 7-AAD+, and CD11b+/7-AAD+ NB4 cells under no treatment, 0.5µM ATO, 1µM ATRA, or combination treatment conditions at 168h of continuous treatment. Stacked bar graphs summarize the flow cytometry analysis of percentage dead (7-AAD+) and differentiated (CD11b+ or CD11b+/7-AAD+) NB4 cells induced by 0.5µM ATO, 1µM ATRA, or combination treatment after 168h of continuous treatment (n=4, error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons).

3.11 Only ATRA and ATO combination treatment sustains differentiation and cell death of NB4 cells 96h post treatment termination

While ATRA alone induces short-term remission in APL patients[18], the complete remission induced by the ATRA and ATO combination treatment regimen suggests long-term lasting effects have been elicited which sustained post treatment termination. To study these long-term effects, we treated the APL cell line NB4 with 1μM ATRA and 0.5μM ATO alone or in combination for 72h and then treatment was terminated by washing the cells and subsequently culturing the cells for an additional 96h in treatment-free medium. The potential synergistic benefits of ATRA and ATO combination treatment became most evident after treatment was terminated for 96h (Figure 8). Ninety-six hours post treatment termination, most of the cells treated with the single agents now lacked staining for the differentiation and death markers (Figure 8). This was in sharp contrast to the combination treated cells that were mostly differentiated and/or dead (CD11b/7-ADD positive cells, Figure 8). This data mimics the clinical findings, whereby ATRA and ATO combination therapy result in sustained effects which persist after the termination of therapy[74,91,92].

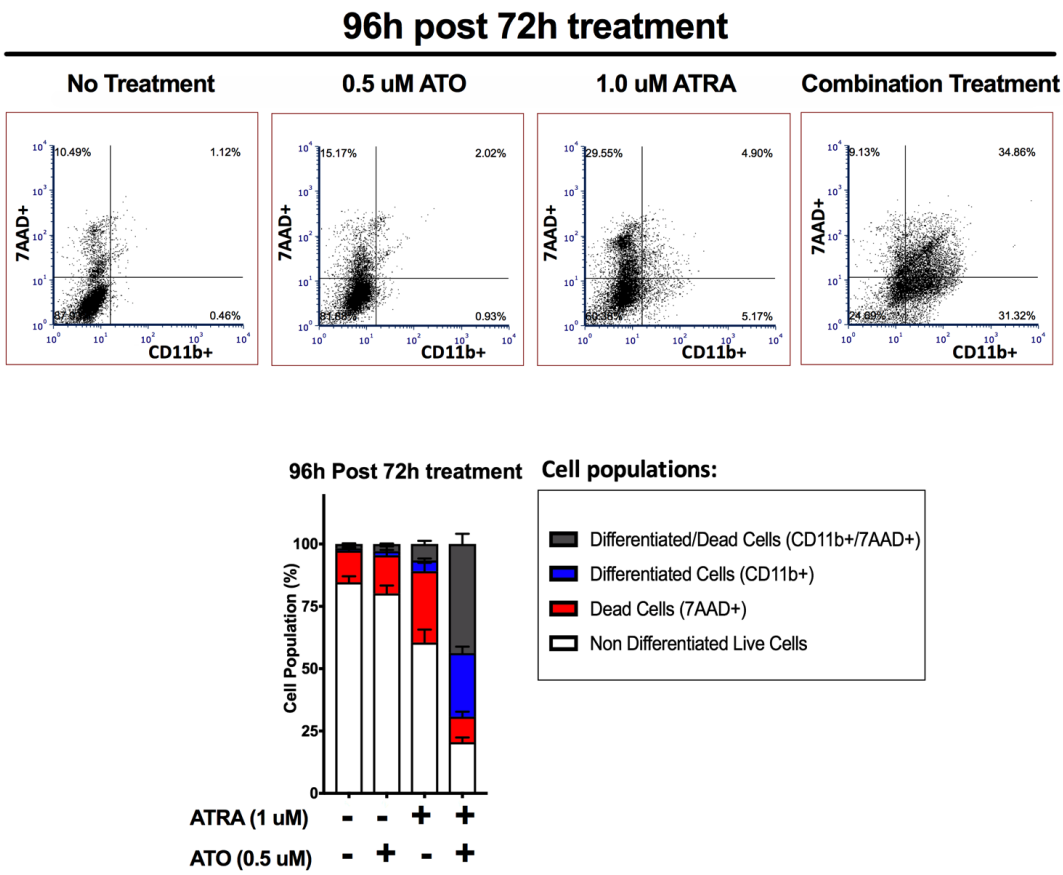


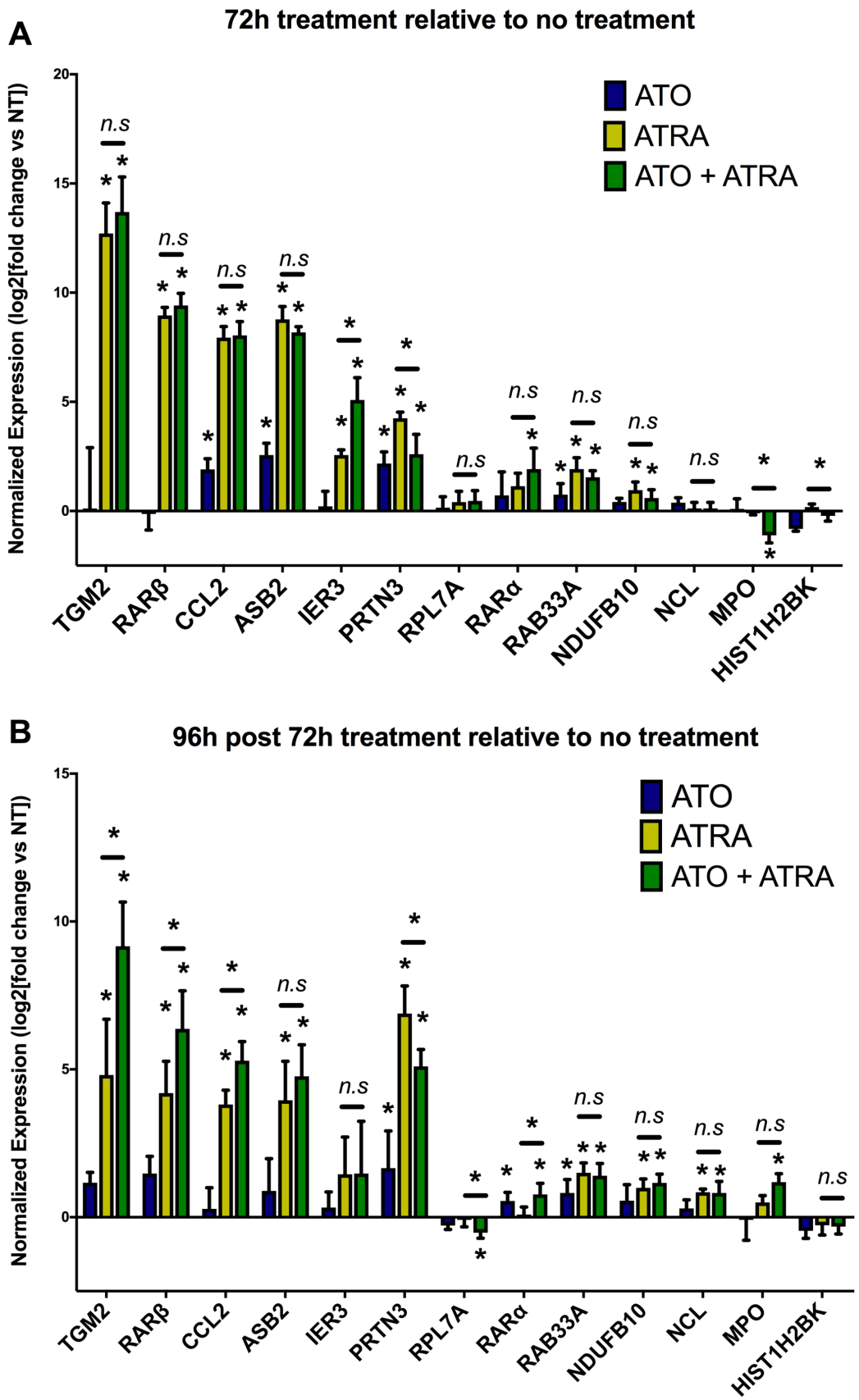
Figure 8. Combination ATRA and ATO treatment sustains differentiation and death of NB4 cells 96h post treatment termination. Representative flow cytometry dot plots for CD11b+, 7-AAD+, and CD11b+/7-AAD+ NB4 cells 96h post treatment termination after 72h of treatment. Stacked bar graphs summarize the flow cytometry analysis of percentage dead (7-AAD+) and differentiated (CD11b+ or CD11b+/7-AAD+) NB4 cells induced by 0.5μM ATO, 1μM ATRA, or combination treatment after 72h treatment and subsequent 96h post treatment termination (n=4, error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons).

3.12 Combination treatment of ATRA and ATO is more effective at maintaining high transcript levels of TGM2, RARβ and CCL2 96h post treatment termination.

Having observed that only ATRA and ATO combination treatment maintain the majority of NB4 cells in a state of terminal differentiation after treatment was terminated (Figure 8), we next wondered if gene expression changes were similarly more persistent. Gene expression changes are a key component of the interfered differentiation and symptoms of APL disease[40]. Supra-physiological levels of ATRA can restore the expression of epigenetically silenced target genes in APL cells[13]. Using QPCR, we determined the effect of ATRA and ATO individually or in combination at 72h and following 96h post treatment termination, on the mRNA levels of several genes involved in APL processes such as leukemic differentiation (TGM2, RARβ), granulocyte function (MPO, PRTN3) and other ATRA-regulated targets (e.g., CCL2) in comparison to single agent treatment[15,40]. ATRA alone and combination treatment resulted in significantly higher transcript levels of several genes (TGM2, RARβ, CCL2, ASB2, RPL7A, RARα, RAB33A, NDUFB10, NCL and HIST1H2BK) after 72h of treatment (Figure 9A). ATO treatment alone had comparatively minor effect on expression of the genes. Notably, for most of the genes, there was no significant difference in the transcript levels induced by ATRA alone and combination treated cells (Figure 9A). In contrast, 96h after termination of treatment, significantly higher transcript levels of TGM2, RARβ and CCL2 were present in cells treated by the combination versus ATRA alone (Figure 9B). Therefore, the combination treatment maintained greater transcript levels of these genes once treatment had been terminated. The sustained expression of these genes may explain why terminal granulocytic differentiation is sustained in combination treated cells in

comparison to single agent treated cells, especially considering the key role that ATRA-induced TGM2 expression has in differentiation of NB4 cells[93].

Figure 9. Combination treatment of ATRA and ATO is more effective at maintaining high levels of TGM2, RARβ and CCL2 mRNA 96h post treatment termination. (A and B) QPCR analysis detects relative levels of mRNA of ATRA target genes in NB4 cells 72h after treatment **(A)** and subsequent 96h post treatment termination **(B)** with 0.5μM ATO, 1μM ATRA, or combination treatment. mRNA levels of target gens are log2 transformed and relative to the no treatment sample and reference genes (n=4, error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons, p value < 0.05 indicated by *).


A
72h treatment relative to no treatment
Normalized Expression (log2[fold change vs NT])
ATO
ATRA
ATO + ATRA
n.s
TGM2
RARβ
CCL2
ASB2
IER3
PRTN3
RPL7A
RARα
RAB33A
NDUFB10
NCL
MPO
HIST1H2BK
B
96h post 72h treatment relative to no treatment
Normalized Expression (log2[fold change vs NT])
ATO
ATRA
ATO + ATRA
n.s
TGM2
RARβ
CCL2
ASB2
IER3
PRTN3
RPL7A
RARα
RAB33A
NDUFB10
NCL
MPO
HIST1H2BK

3.13 ATRA induces sustained enrichment of H3K9/14ac at the TGM2 and RARβ promoters in NB4 cells, which is not augmented by combination treatment.

Decreased H3K9/14ac at target genes is key in their decreased expression in APL cells and ATRA-induced gene expression is associated with H3K9/14ac enrichment[49]. We therefore wondered if the greater sustained expression post treatment termination of some target genes induced by combination treatment (Figure 8B), was due to greater enrichment of H3K9/14ac. Since *TGM2* and *RARβ* were the most induced, with the greatest sustained expression by the combination treatment once treatment was terminated, we focused on these two genes for H3K9/14ac analysis by ChIP-qPCR. In agreement with previous reports, ATRA induced H3K9/14ac enrichment at *TGM2* and *RARβ* promoters (Figure 10A). Combination treatment induced similar levels of H3K9/14ac enrichment at both 72h and after 96h post treatment termination. The lack of significant difference between the combination treatment versus the ATRA treatment alone (Figure 10A), suggests that H3K9/14ac enrichment likely does not play a role in the greater sustained expression of the genes induced by combination treatment (Figure 8B).

We next wondered if perhaps the combination treatment decreases repressive H3K9me3 and H3K27me3 marks associated with silencing of *TGM2* and *RARβ* in APL cells[13]. Consistent with previous reports, ATRA had a minimal effect on H3K9me3 and H3K27me3 enrichment at the target genes (Figure 10B and C). The combination treatment also had insignificant effects on the enrichment of the two repressive histone marks (Figure 12B and C). Overall, this indicates that the greater sustained TGM2 and RARβ mRNA levels induced by combination treatment (Figure 8B) is not due to changes in histone modifications (Figure 10).

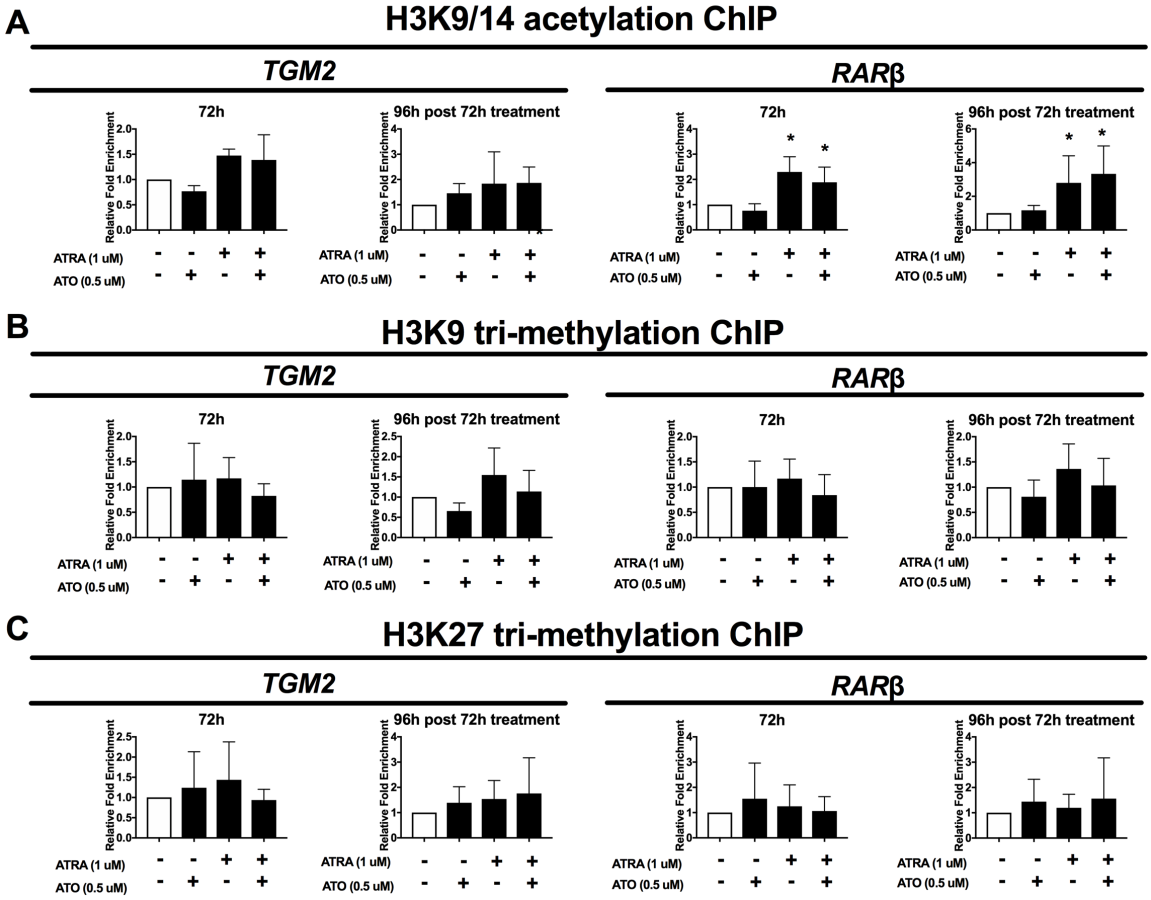


Figure 10. ATRA and combination treatment induce sustained enrichment of H3K9/14ac at *TGM2* and *RARβ* promoters in NB4 cells. H3K9/14ac **(A)**, H3K9me3 **(B)**, and H3K27me3 **(C)** enrichment at *TGM2* and *RARβ* promoters as measured by QPCR following ChIP with antibodies specific to the histone modification in NB4 cells following 72h of treatment and subsequent 96h post treatment termination (n=4, error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons, p value < 0.05 indicated by *).

3.14 Overall H3K9ac levels are significantly lowered under ATRA treatment after 72h treatment, and under combination treatment 96 hours post treatment termination.

We next wondered if the enrichment of H3K9ac with TGM2 and RAR*β* was due to an overall increase in that particular histone modification instead of being specifically enriched at those specific gene regions. For this purpose, we performed western blots to assess the total levels of H3K9ac in the cells at the various treatment conditions. Interestingly, the overall levels of H3K9ac was significantly reduced by ATRA treatment alone after 72 hours (Figure 11), in contrast to its enhanced enrichment with TGM2 and RAR*β.* Additionally, 96 hours after treatment had been terminated, the levels of H3K9ac was significantly reduced under the combination of ATRA and ATO and remained reduced under single ATRA treatment (Figure 11). Despite this reduction in overall levels of H3K9ac96 hours after treatment had been terminated, the promoter region of TGM2 and RAR*β* were significantly enriched with this histone modification at this time point (Figure 10), therefore the histone enrichment is gene specific.

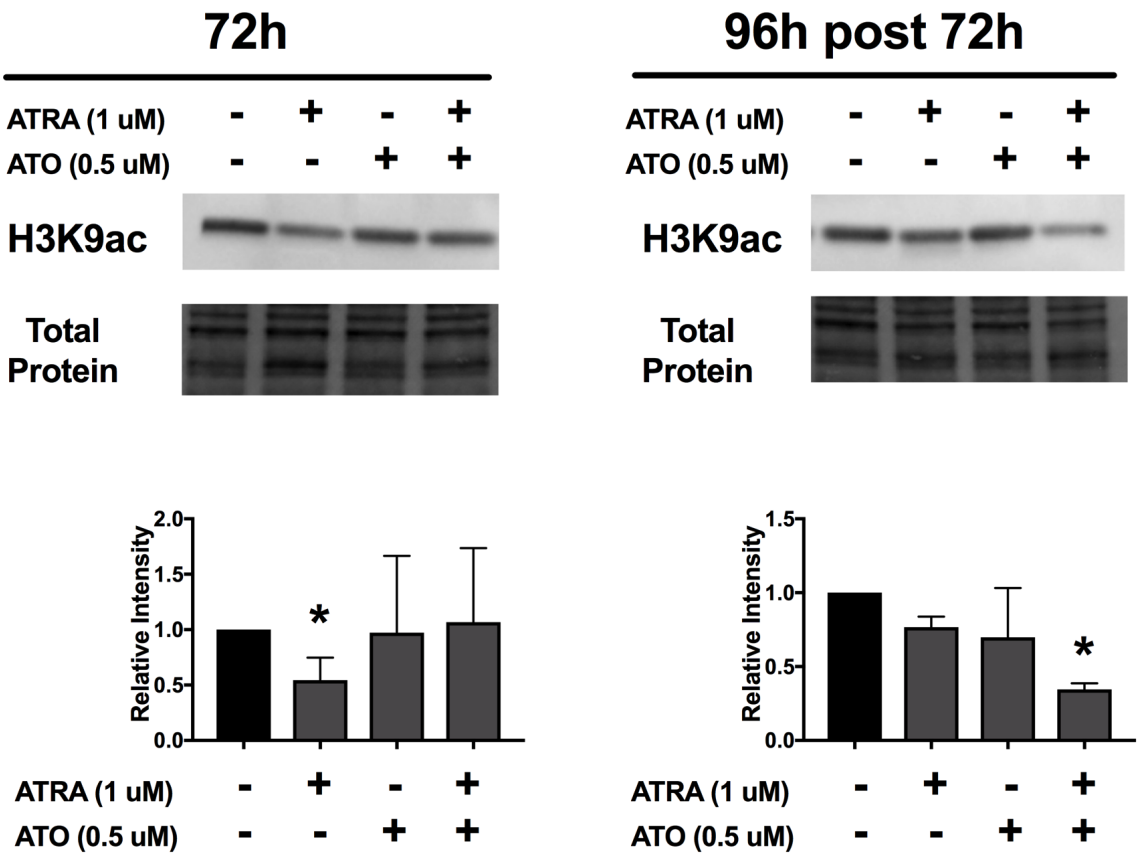


Figure 11. ATRA alone and in combination decreases global levels of H3K9ac. Global levels of H3K9/14ac were reduced under ATRA treatment after 72h and combination treatment under combination ATRA and ATO, as measured by western blotting analysis with antibodies specific to the histone modification in NB4 cells following 72h of treatment and subsequent 96h post treatment termination (n=3, error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons, p value < 0.05 indicated by *).

3.15 H3K9me3 levels are significantly higher under combination treatment after 72h, however this increase is lost 96 hours post treatment termination.

Decreased levels of repressive histone modifications suggests that transcriptional activity is enhanced. Since we did not observe increased enrichment of TGM2 and RARβ with the repressive histone modifications H3K9me3 and H3K27me3 under combination treatment (Figure 10), we next wanted to investigate whether combination treatment can decrease overall levels of these repressive histone modifications by western blotting. Although the level of H3K9me3 was increased under combination treatment after 72 hours, this increase was lost 96 hours after treatment had been terminated (Figure 12); therefore, it likely does not play a role in the sustained differentiation induced by the combination treatment.

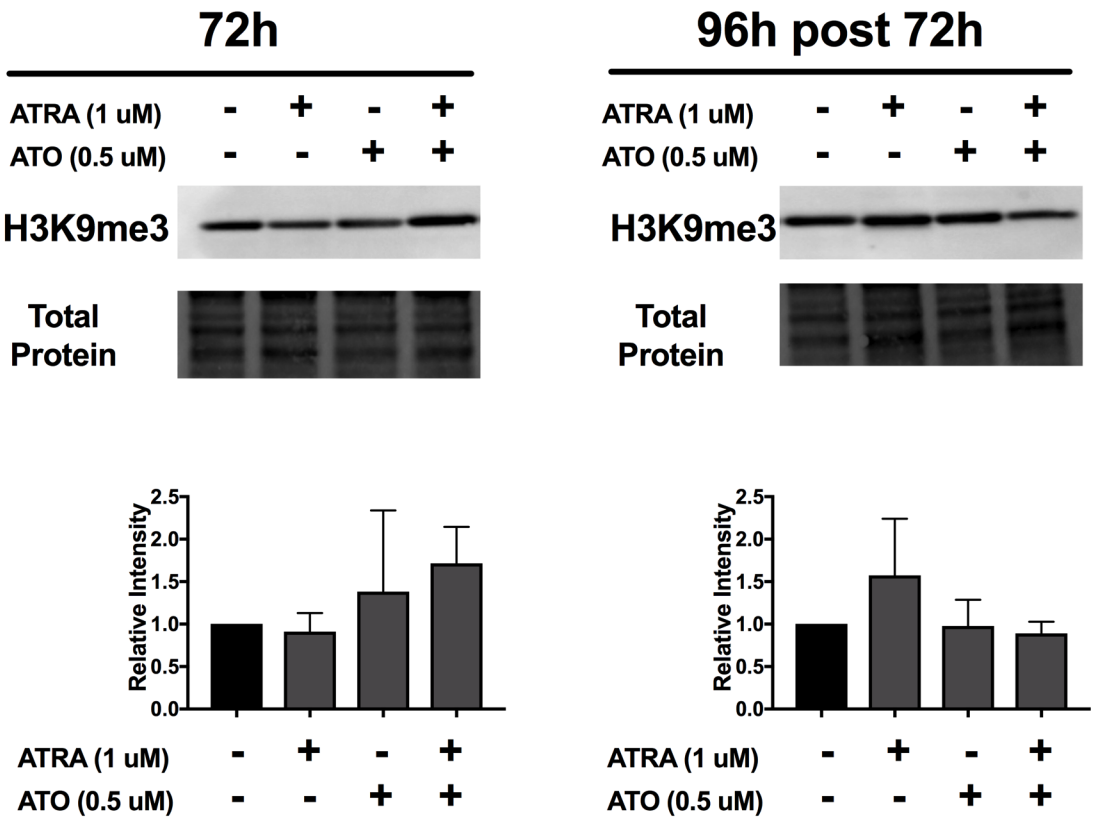


Figure 12. ATRA alone and in combination induces changes in global levels of H3K9me3. Global levels of H3K9me3 were significantly increased under combination treatment after 72h however this increase was lost 96h post 72h treatment, as measured by western blotting analysis with antibodies specific to the histone modification in NB4 cells following 72h of treatment and subsequent 96h post treatment termination (n=3, error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons, p value < 0.05 indicated by *).

3.16 Global H3K27me3 levels remain unchanged by ATRA and ATO alone and in combination

We also wanted to assess the global levels of the repressive H3K27me3 mark by western blotting, which was not enriched with TGM2 or RARβ. Similarly, H3K27me3 levels are not significantly changed under combination treatment after 72 hours and 96 hours post treatment termination in comparison to single agent treatment and no treatment conditions (Figure 13). Therefore, combination treatment does not affect repressive histone modification levels at the global level in addition to gene specific interactions.

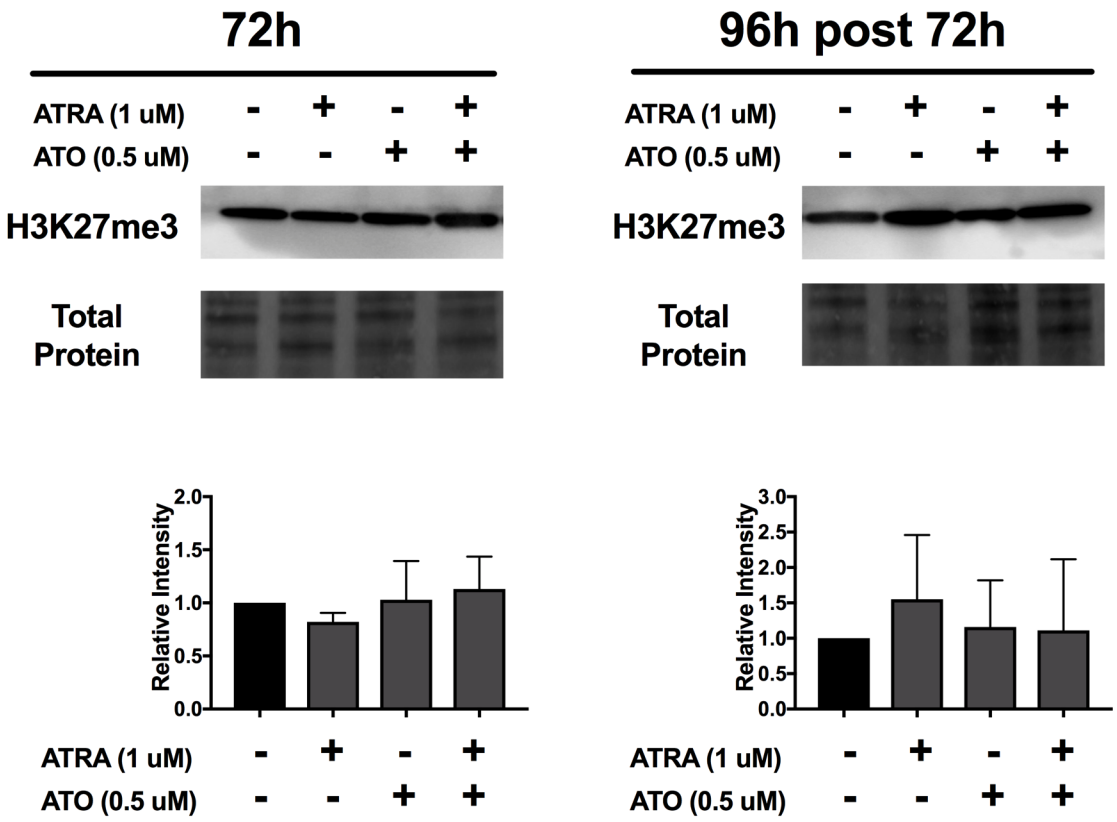


Figure 13. Global levels of H3K27me3 remain unchanged under single agent and combination treatment. Global levels of H3K27me3 were not significantly elevated in any treatment conditions at both time points, as measured by western blotting analysis with antibodies specific to the histone modification in NB4 cells following 72h of treatment and subsequent 96h post treatment termination (n=3, error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons, p value < 0.05 indicated by *).

3.17 Combination treatment significantly reduces DNA methylation of the CpG sites in the promoter regions of TGM2 and RARβ in NB4 cells.

CpG island DNA hypermethylation silencing of genes contributes to the arrested differentiation of granulocytes in APL[16]. Although ATRA is capable of inducing APL cells into a differentiated granulocytic phenotype, most evidence suggest that target genes, including differentiation-inducing gene *TGM2* and canonical target gene *RARβ* remain hypermethylated[14,41]. However, we wondered if the combination treatment could be affecting the promoter methylation of the genes, contributing to the greater sustained transcript levels of the genes post treatment termination. Bisulfite pyrosequencing was used to interrogate the 20 CpGs in the CpG island in the *TGM2* promoter (Figure 14A). Combination treatment significantly reduced the total C-methylation of the *TGM2* promoter region after 72h treatment (Figure 14B), which was sustained 96h after treatment had been terminated (Figure 14C). In contrast, treatment with ATRA or ATO alone was insufficient to significantly reduce methylation of the region. Similarly, methylation of the 15 CpGs in the CpG island neighboring the *RARβ* transcription start site was quantified using bisulfite pyrosequencing (Figure 15A). Again, treatment with ATRA or ATO alone did not significantly alter CpG methylation, but combination treatment significantly reduced the total C-methylation of the *RARβ* promoter region at 72h (Figure 15B) and this was sustained 96h after treatment termination (Figure 15C). Together, this provides new evidence showing that the combination of ATRA and ATO reduces the aberrant methylation of key target genes, and similar to the analysis of transcript levels (Figure 4B), this effect was sustained beyond treatment termination (Figures 14 and 15).

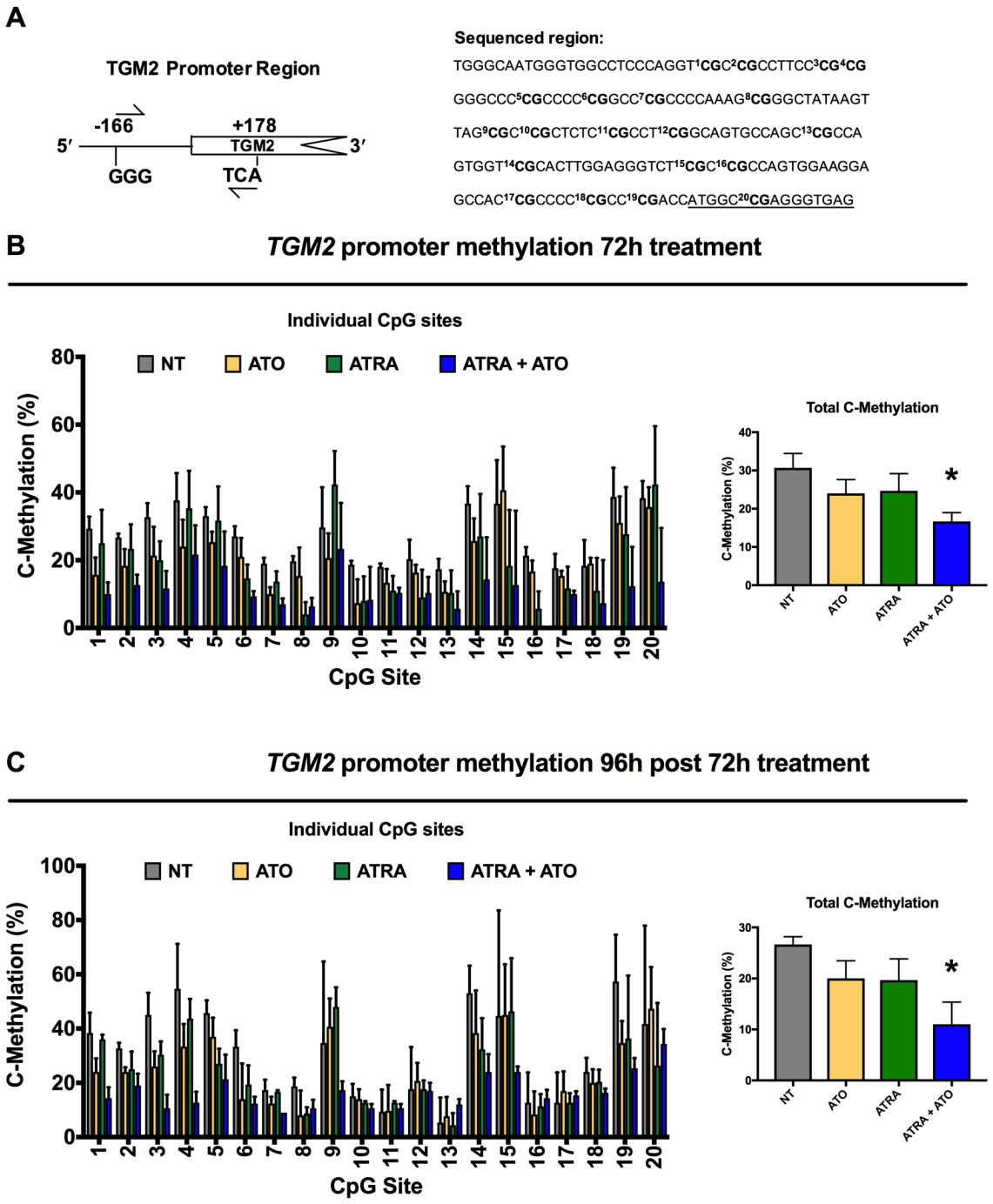


Figure 14. Combination treatment significantly reduces DNA methylation of the CpG island in the promoter region of *TGM2* in NB4 cells. (A) Schematic representation of the *TGM2* promoter region and the specific 20 CpG sites located within the region that was bisulfite pyrosequenced. **(B and C)** The methylation percentage of the individual 20 CpG sites and total C-methylation percentage of the region in NB4 cells following 72h of treatment **(B)** and subsequent 96h post treatment termination **(C)**, (n=3, error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons, p value < 0.05 indicated by *).

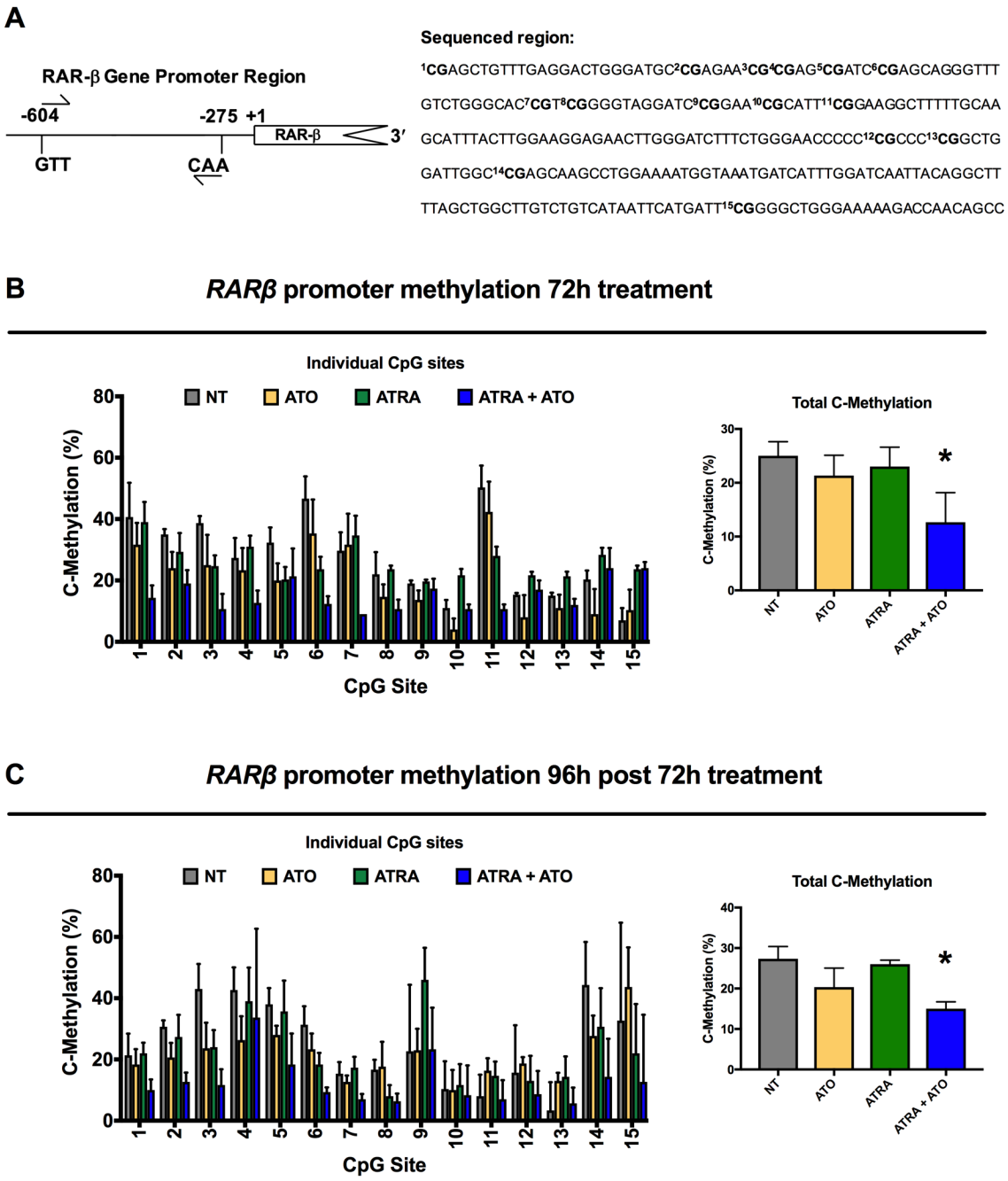


Figure 15. Combination treatment significantly reduces DNA methylation of the CpG island in the promoter region of *RARβ* in NB4 cells. **(A)** Schematic representation of *RARβ* and the specific 15 CpG sites located within the region that were bisulfite pyrosequenced. **(B and C)** The methylation percentage of the individual 15 CpG sites and total C-methylation percentage of the region in NB4 cells following 72h of treatment **(B)** and subsequent 96h post treatment termination **(C)**, (n=3, error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons, p value < 0.05 indicated by *).

3.18 Global DNA methylation in APL NB4 cells are below detection threshold of MethylFlash Methylated DNA Colormetric Quantification Kit.

Global levels of DNA methylation can be hypermethylated or hypomethylated in diseases such as cancers[95]. In APL, DNA methylation levels are elevated in comparison to non-APL control, however the DNA methylation changes remain unchanged under ATRA treatment alone[95]. Therefore, we wanted to see whether the combination with ATO, which has been demonstrated to exert a demethylating effect on several APL cell lines, could induces changes in the overall level of DNA methylation. Additionally, we also wondered if the effect of combined ATRA and ATO treatment on CpG methylation extended beyond the target genes and instead was due to a genome-wide effect. In order to measure the DNA methylation of our treated APL NB4 samples, we used the MethylFlash Methylated DNA Colormetric Quantification Kit that measures levels of 5-methylcytosine (5-mC) in an ELISA-like microplate-based method. I generated a standard curve using the kit-supplied positive control (synthetic DNA containing 50% of 5-methylcytosine, Figure 16A). This also established the minimum threshold of DNA methylation the assay can detect accurately (Figure 16A). In comparison to the standard curve generated from the positive control, the methylation values of the APL NB4 samples at 72h (Figure 16B) and 96h post 72h treatment (Figure 16C) were under the minimum threshold of detection and the readings fell outside the linear range of the standard curve. Therefore, the MethylFlash Methylated DNA Colormetric Quantification Kit is not sensitive enough to measure the methylation value of these samples. A more sensitive method was required to measure global DNA methylation levels in NB4 cell samples.

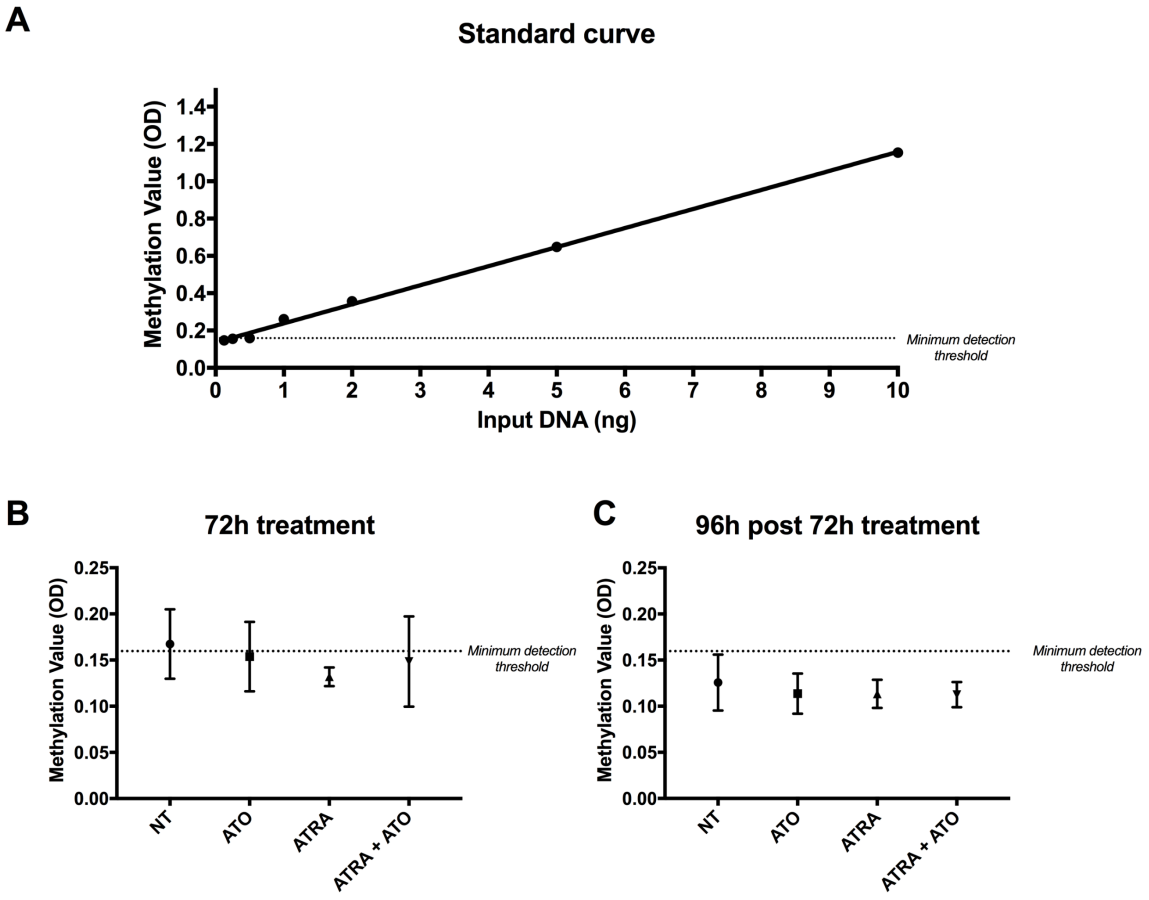


Figure 16. Global DNA methylation in APL NB4 cells fall below detection threshold measured by the MethylFlash Methylated DNA Colormetric Quantification Kit. (A) The standard curve generated from the OD450 values of serial dilutions of the kit-supplied DNA methylation standard, ranging from 10ng to 0.125ng. The dotted line indicates the minimum threshold of detection of the assay. **(B and C)** The methylation values of the NB4 cells under all 4 treatment conditions: no treatment, 1.0μM ATRA only, 0.5μM ATO only and combination 1.0μM ATRA and 0.5μM ATO falls under the minimum detection threshold of the colorimetric assay, at 72h and 96h post 72h treatment (n=3, error bars represent standard deviation).

3.19 Global DNA methylation levels, represented by bisulfite pyrosequencing of LINE-1, are unchanged in ATRA, ATO or combination treated NB4 cells.

Since the colourmetric assay was insufficiently sensitive to quantify overall DNA methylation in the NB4 cells, an alternative method, more sensitive method based on bisulfite pyrosequencing was used. Assessing the methylation of long interspersed nucleotide element 1 (LINE-1) is an accepted surrogate for general global methylation levels of the genome[96]. LINE-1 elements are transposable repetitive elements making up 17% of genomic DNA and are often heavily methylated, holding up to a third of the methylation in the genome. We performed bisulfite pyrosequencing to assess methylation levels of 27 CpG sites within the promoter region of LINE-1 subfamily L1PA2 (Figure 17A)[42,45]. LINE-1 methylation was unchanged by any of the treatment conditions at 72h or 96h after treatment was terminated (Figure 17B). This suggests that the reduced CpG methylation in response to combination treatment is associated with target genes (Figures 14 and 15) and is not global (Figure 17).

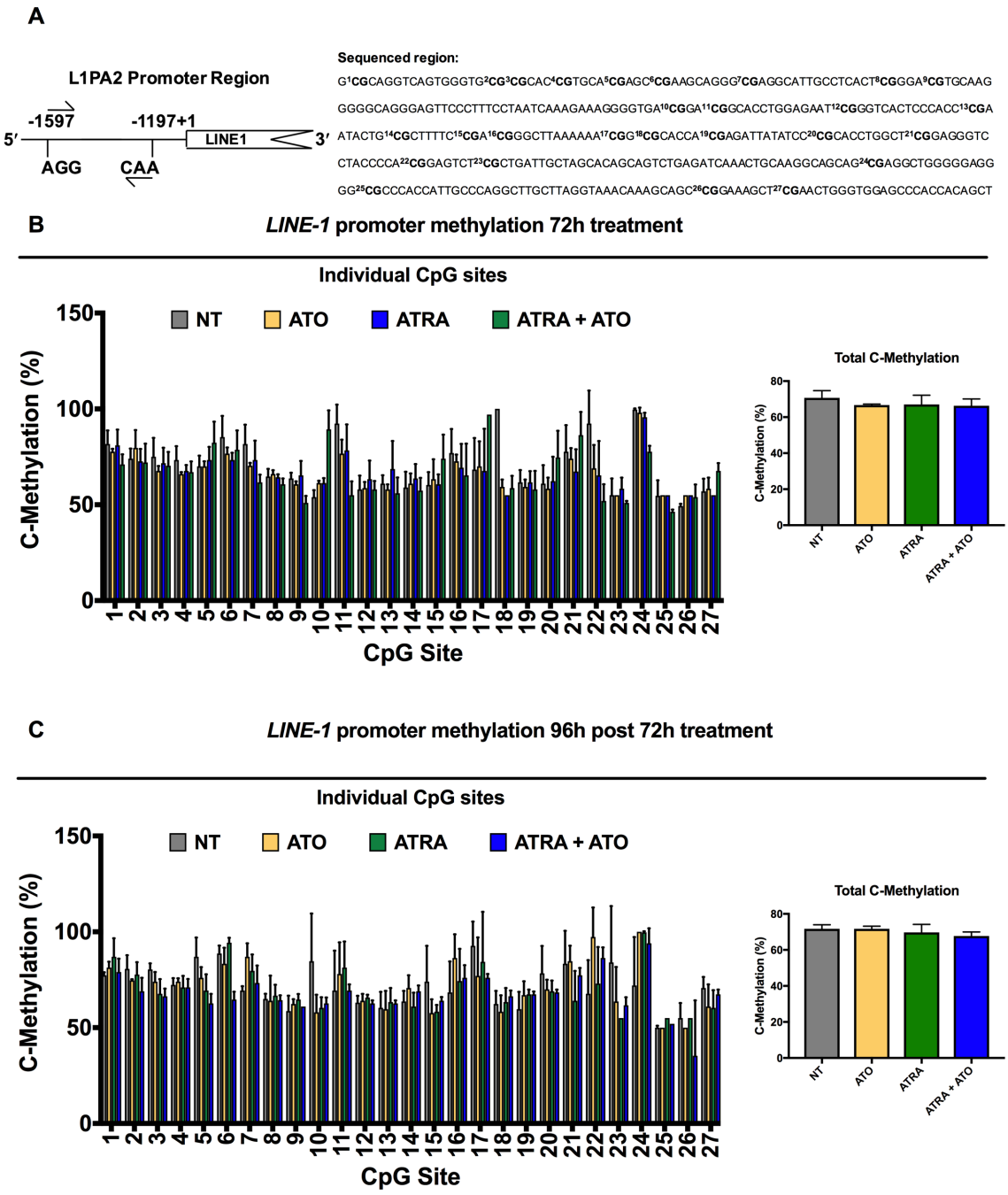


Figure 17. Global DNA methylation levels, represented by bisulfite pyrosequencing of *LINE-1*, are unchanged in ATRA, ATO or combination treated NB4 cells. (A) Schematic representation of the *LINE-1* subfamily *PA2* and the 27 CpG sites located within the region that were bisulfite pyrosequenced. **(B and C)** The methylation percentage of the individual 27 CpG sites and total C-methylation percentage of the region in NB4 cells following 72h of treatment **(B)** and subsequent 96h post treatment termination **(C)**, (n=3, error bars represent standard deviation, significance determined using one-way ANOVA with multiple comparisons, p value < 0.05 indicated by *).

CHAPTER 4 DISCUSSION

4.10 Preface

Despite the drastically improved outcome for patients with APL currently, treatment failure in patients treated with ATRA and chemotherapy remains a risk due to the complications associated with chemotherapy treatment. The potential for chemotherapy-free treatment with the induction agents ATRA and ATO to become the standard front-line treatment is highly appealing; however, there remains a lack of understanding of how this combination treatment works. Clinically, combined ATRA and ATO therapy is curative in APL patients, inducing long-lasting remission and improved overall survival, whereas ATRA treatment alone is effective at eliciting only short-term remission and is susceptible to disease relapse[29–33,46].

Similar to other diseases, APL is characterized by an aberrantly modified epigenome, specifically at the DNA methylation and histone modification level which are important for disease development and response to treatment. Previously, single agent ATRA and ATO have been shown to influence histone modifications and DNA methylation respectively; however, the epigenetic changes induced by the combination of both these agents in APL has not been investigated. Importantly, these potential changes induced by combination treatment could be responsible for the sustained and long-term effect that combination treatment induces, but single agent treatment cannot achieve. In this study, I found that the combination of ATRA and ATO induced enhanced differentiation and cell death of APL cells that is sustained after treatment had been terminated (Figure 18A). These combination-treated cells maintain high transcript levels of TGM2 and RARβ (Figure 18B) which are associated with gene specific decreased

DNA methylation (Figure 18C), a permissive epigenetic change not seen in single agent treated cells. Therefore, the effect of combination treatment on the epigenome may be due more to gene specific effects, and not global epigenetic changes.

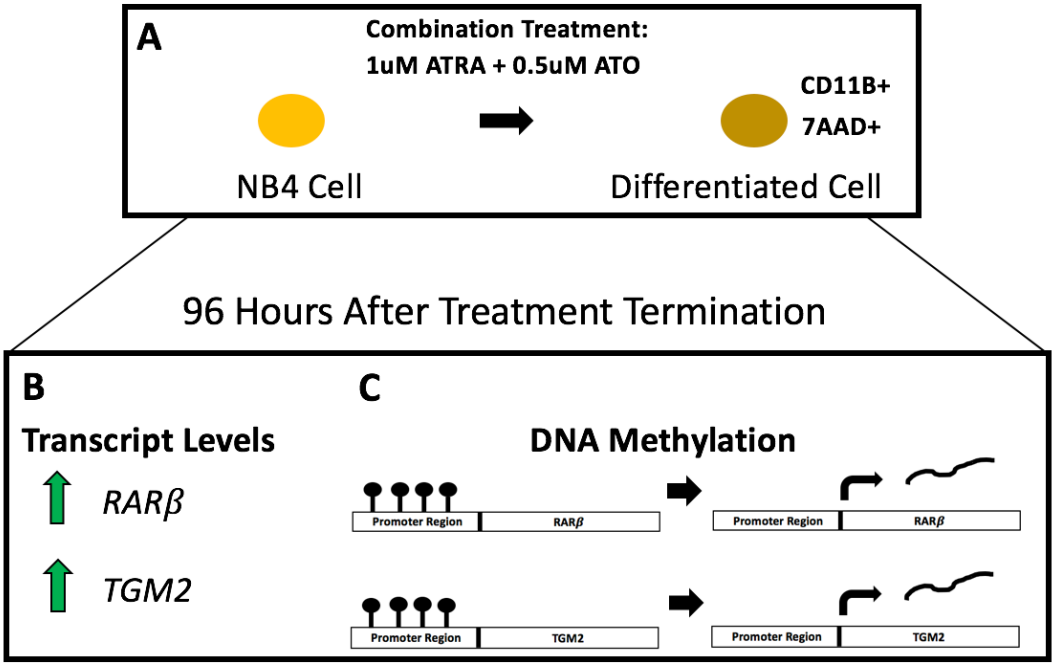


Figure 18. ATRA (1uM) and ATO (0.5uM) in combination induces APL NB4 cell differentiation, which coincides with elevated transcript levels of TGM2 and RARβ, and decreased methylation. (A) Combination treated cells maintain terminal differentiation characterized by expression of CD11B and 7AAD 96 hours after 72 hour treatment, which is not seen in single agent treated cells. **(B)** Transcript levels of TGM2 and RARβ are higher in combination treated cells in comparison to single agent treated cells. **(C)** The CpG sites located within the promoter regions of TGM2 and RARβ have significantly decreased levels of methylation in comparison to single agent treated cells.

4.11 ATRA and ATO in combination induces significantly increased granulocytic differentiation, even after treatment termination

Previous studies of cultured APL cells describe the enhanced differentiation/cell death induction of the combination treatment over ATRA alone; however, these studies do not extend the analyses post treatment termination[37,47,48]. This study compares, for the first time, the effects of these treatments four days after treatment termination. Unexpectedly, we observed that under these conditions the effects of the combination treatment were more dramatic. Initially, we investigated increasing concentrations of ATRA up to 1.0μM and ATO up to 0.5μM to determine the degree of granulocytic differentiation induced by either agents alone and in combination. ATRA at 1.0μM induced granulocytic differentiation in the short-term (72h) and was increased further after long-term continuous treatment (168h); however, ATRA-induced effects were mostly lost once treatment had been terminated (Figure 8). This is in sharp contrast to the combination treatment, which resulted in mostly differentiated and/or dying cells (Figure 8). These findings model the long-lasting effects induced by the combination therapy in patients, which ATRA alone fails to do.

4.12 ATRA and ATO in combination maintain high transcript levels of key differentiation genes associated with demethylation

Due to the normal role of RARα in regulating transcription of genes, where its response to retinoic acid results in relinquished gene repression and transcriptional activation, I wanted to test whether combination treatment restored expression of genes silenced during APL pathology and whether these genes were maintained once treatment was terminated. The sustained expression of the genes identified after treatment has been terminated could explain why patients treated with combination treatment in the clinic stay

in remission without experiencing relapse. Significantly, TGM2 and RARβ transcript levels were higher under combination treatment in comparison to single agent treatments. The sustained expression of these genes corresponds to the enhanced differentiation status of the NB4 cells under combination treatment, which suggests that permanent transcriptional changes were exclusively induced.

A potential mechanism for the higher transcript level could be due to permissive epigenetic modifications, which would have a more permanent effect on increasing gene expression. Previous reports indicate that key differentiation genes such as *RARβ* and *TGM2* remain methylated in NB4 cells, despite ATRA induced differentiation[105,106]. This encouraged us to evaluate the epigenetic modifications of these genes. Epigenetic modifications such as histone marks and DNA methylation regulate gene transcription and are aberrant in APL resulting in silencing of many target genes[16,41]. Although the level of global methylation in APL NB4 cells remained unchanged under combination treatment (Figure 17), an important contributing factor to the increased effectiveness of combination treatment may be demethylation of target genes that are aberrantly methylated in APL (Figures 14-15). The aberrant methylation and silencing of *RARβ* is particularly well described in APL[14,15,41,49]. With the exception of one early study that used methylation-specific PCR to quantify *RARβ* methylation post ATRA treatment[49], other later studies using more quantitative techniques report that ATRA treatment alone fails to revert the aberrant methylation of *RARβ* and other genes, including *TGM2*[14,15,41]. Using the quantitative bisulfite pyrosequencing technique, our results are in agreement with the later studies, where 1μM ATRA does not significantly alter CpG methylation of target genes in APL cells (Figures 14-15)[14,15,41]. Along with its importance in granulocyte differentiation, TGM2 is partly responsible for differentiation syndrome caused by ATRA treatment[93]. It

is possible that the enhanced expression of this gene under combination treatment could also account for why combination treatment results in differentiation syndrome.

In contrast, with respect to ATO, there are reports of its effects on DNA methylation in APL cells. A recent study showed that 2.0μM ATO reduced DNA methylation and increased mRNA levels of cell cycle related genes in NB4 cells[50]. ATO reduced transcript levels of DNA methyltransferases 1, 3A and 3B in NB4 cells, which resulted in genome-wide demethylating effects on DNA[50]. This is consistent with another study on the cell line HL-60 (an APL-like cell line that lacks the PML-RARα fusion), in which 1μM ATO modestly reduced global methylation[51]. This suggests that unlike ATRA[14,15,41], 1-2 μM ATO has genome-wide DNA demethylating effects in APL cells[50,51]. In our study ATO did not reduce global methylation or target gene-specific methylation in NB4 cells; however, this could be due to the lower 0.5μM ATO that was applied to the cells (Figures 14-15, 17). It is noteworthy, the transcript levels of target genes in NB4 cells treated with ATO alone were comparably modest (Figure 9), consistent with the negligible effects that 0.5μM ATO had on histone modifications (Figure 10) and DNA methylation (Figures 14-15).

4.13 ATRA and ATO in combination increase enrichment of key differentiation genes with permissive histone modifications

In the literature, the target binding sites of PML-RARα are associated with decreased association with the permissive histone modifications H3K9ac and H3K9/14ac in addition to increased methylation[110]. Pharmacological doses of ATRA has been reported to increase H3 acetylation without affecting DNA methylation however the effect of combination treatment has not been studied[110]. Since our study identified that combination treatment maintained high transcript levels of the key differentiation genes TGM2 and

RARβ and these were associated with demethylation, I next wanted to study the effect of the combination treatment on the interaction of these transcripts with the permissive histone modification. Utilizing ChIP combined with qPCR, I interrogated the DNA associated with H3K9/14ac and discovered that after 72h of treatment, both *TGM2* and *RAR*β *were* significantly enriched under combination treatment and single ATRA treatment to a similar degree. Ninety-six hours after treatment had been terminated however, RARβ remained significantly enriched with H3K9/14ac under both treatment conditions, while TGM2 was only significantly enriched under combination treatment (Figure 10). Since there was minimal differences between the enrichment caused by combination treatment in comparison to single ATRA at this time point, the difference in maintained transcript levels seen only under combination treatment cannot be explained by a histone modulating effect. The addition of ATO to ATRA in the combination treatment therefore does not augment the enrichment of the transcripts with the permissive histone modification.

4.14 Limitations of study

Although the NB4 cell line is a representative APL cell line which makes it an ideal tool to study APL, there are several issues with using this as the sole model in this study. A population of NB4 cells are homogenous with low expression of CD11b and the lack of mature granulocytic markers, which makes it ideal to study APL; however, this does not represent the hematopoietic stem cell niche, which is more diverse in the types of cells present, even in APL patients. A bone marrow sample extracted from a patient would demonstrate a predominantly leukemic promyelocyte population, but there would still be some other cells present. Therefore, assessing the global methylation levels of a population

of NB4 cells under a certain treatment condition, which was done in this study, in comparison to assessing the global methylation levels of a patient sample under the same treatment condition could have completely different changes in the global methylation levels. This could be due to the presence of other cells that could be hypermethylated or hypomethylated. Additionally, although NB4 cells have similarities with APL patient samples in their gene expression profiles, they also have differences in upregulated and downregulated genes[110]. The changes induced by combination treatment in the NB4 cells could be different than in patients. Finally, the immortalization process involved with transforming the NB4 cell lines could potentially have effects on the epigenetic landscape of these cells. In the literature, the presence of PML-RARα alone is not enough to induce changes at the DNA methylation level, so the aberrant DNA methylation in APL patients is not caused by that translocation, but a later effect of leukemogenesis[95]. Thus, there could be other factors that are not present in the NB4 cells or that were lost during the immortalization process, potentially making them not representative.

Another limitation to the study was the use of assays that measured global levels of histone modification and DNA methylation to assess potential changes caused by ATRA and ATO. Epigenetic changes can be dynamic in nature, where the total overall levels may stay static but the specific changes are what is important.

Additionally, the list of genes of interests in the qPCR analysis (Figure 9) was generated in a biased manner and based on the current literature that identified potential genes involved in APL. Therefore, other potential genes that could be differentially expressed by combination treatment in comparison to single agent treatments were not assessed, making the scope of the study narrow.

4.15 Future Directions

The results from these studies highlight the need to perform genome-wide transcriptome, ChIP-seq of histone modifications, and methylome analyses of combination ATRA and ATO treated NB4 and patient APL cells. Thus far, genomic analyses have been primarily focused on the effects of ATRA only in APL cells[15,16]; however, with the increasing utilization of ATRA and ATO in the clinic, the effects of the combination of ATRA and ATO on the epigenome and transcriptome needs to be understood. Identifying changes exclusively caused by combination treatment at the transcriptome level can reveal important genes that need to be expressed or silenced in APL pathology, which could potentially be targeted with the advent of new gene editing techniques such as CRISPR[111]. This could potentially both improve the success and even replace this combination therapy. Additionally, characterizing the DNA methylation pattern caused by combination treatment at a global level could identify other genes that are demethylated exclusively by the combination treatment similarly to *TGM2* and *RARβ*.

To confirm our results, the next step would be to repeat these experiments on patient samples to corroborate the effects of combination identified in this study. Additionally, the use of the transgenic APL murine model previously described could be used to confirm the sustained effects of the combination treatment on the leukemic cells after terminating treatment in an *in vivo* model[77]. This would also enable the assessment of the improved effect of combination treatment on overall survival of the animal.

Ultimately, the differences in the effects of combination treatment may be especially crucial for APL patients who are resistant to combination therapy and experience disease relapse[54]. An increased understanding of the role of epigenetics in APL treatment response may help in the development of novel strategies to overcome this risk of treatment

failure. This includes the use of demethylating agents such as decitabine or histone decacetylase inhibitors such as vorinostat, which may improve on the DNA demethylating effects demonstrated in our study[55]. Decitabine has been assessed in combination with ATRA in a phase II study where it achieved favorable effects and has been characterized to cause gene specific demethylation and increased histone acetylation of the *TNF-related apoptosis-inducing ligand (TRAIL)* gene[114,115]. Therefore it may be worthy to investigate its compatibility with ATRA and ATO.

4.16 Conclusion

ATRA and ATO is effective in the clinic and presents an appealing chemotherapy-free treatment option for APL, however the mechanism behind this has not been elucidated. The epigenome of APL patients is aberrantly altered and important for disease development. Tested here for the first time are the effects of ATRA and ATO combination treatment on the epigenetic modifications of APL cells. In this study, I identified the sustained differentiation and cell death caused only by combination treatment, even after treatment termination, coincides with elevated transcript levels of two genes, *TGM2* and *RARB* that are significantly demethylated to a degree not seen in single agent treatment.

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