EVALUATION OF IMMUNE RESPONSE IN ACUTE PROMYELOCYTIC LEUKEMIA TREATED WITH ARSENIC TRIOXIDE

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DEPARTMENT OF HEMATOLOGY

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FOR

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Thiruvananthapuram

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TO

THE SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM

Thiruvananthapuram

IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

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DECLARATION BY STUDENT

I, Ansu Abu Alex, hereby certify that I had personally carried out the work depicted in the thesis entitled, **"Evaluation of immune response in acute promyelocytic leukemia treated with arsenic trioxide"**.

No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

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This is to certify that **Ms. Ansu Abu Alex**, in the **Department of Hematology, Christian Medical College,Vellore** has fulfilled the requirements prescribed for the Ph.D degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum.

The thesis entitled, **"Evaluation of immune response in acute promyelocytic leukemia treated with arsenic trioxide"** was carried out under my direct supervision. No part of the thesis was submitted for the award of any degree or diploma prior to this date.

Clearance was obtained from the Institutional Ethics committee and Institutional Animal Ethics for carrying out the study.

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‘*Success is the sum of small efforts, repeated day-in and day-out.*

- Robert Collier.

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This thesis is dedicated to the loving memory of my Father

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ABBREVIATIONS

7-AAD	7 aminoactinomycin D
ADCC	Antibody-dependent cell-mediated cytotoxicity
AGE	Agarose gel electrophoresis
aGVHD	acute graft versus host disease
ALC	Absolute lymphocyte count
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ANC	Absolute neutrophil count
APC	Allophycocyanin
APCs	Antigen presenting cell
APL	Acute promyelocytic leukemia
APS	Ammonium persulfate
ATO	Arsenic trioxide
ATRA	All- trans- retinoic acid
BM	Bone marrow
BSA	Bovine serum albumin
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
cDNA	complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
CLIP	Class II associated invariant chain peptide
CLL	Chronic lymphocytic leukemia
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukemia
CMP	Common myeloid progenitor
CR	Complete remission

CTLA-4	Cytotoxic T lymphocyte associated protein 4
DAMP	Damage associated molecular pattern
DC	Dendritic cells
DFS	Disease free survival
DIC	Disseminated intravascular coagulation
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
dNTP	deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
EFS	Event free survival
ELISA	Enzyme-linked immunosorbent assay
FAB	French American British
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
FVB/N	Friend leukemia virus B
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GST	Glutathione S-transferase
GUS	beta-glucuronidase
HAT	Histone acetylase
HMGB-1	High mobility group box 1 protein
HSA	Human serum albumin
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
IDO	Indoleamine 2,3 dioxygenase
IFN	Interferon

IL-15	Interleukin-15
ILC	Innate lymphoid cells
IMDM	Iscove's modified dulbecco's medium
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KIR	Killer cell immunoglobulin-like receptors
LAMP-1	Lysosome associated membrane protein-1
LTS	Long term survivors
MDSC	Myeloid-derived suppressor cells
MEM	Minimum essential media
MEP	Megakaryocyte–erythrocyte progenitor
MGG	May-grünwald giemsa
MHC	Major histocompatibility complex
MIC	MHC class I polypeptide- related sequence
MNC	Mononuclear cells
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCR	Natural cytotoxicity receptors
NK	Natural killer
NO	Nitric oxide
NPM1	Nucleophosmin
OS	Overall survival
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PD-1	Programmed death-1
PE	Phycoerythrin
PerCP	Peridinin chlorophyll
PGE2	Prostaglandin E2
PLZF	promyelocytic leukemia zinc finger protein

PML	Promyelocytic leukemia protein
POD	PML oncogenic domains
PRR	Pattern recognition receptors
RARα	Retinoic acid receptor alpha
RAS	Retinoic acid syndrome
RFS	Relapse free survival
ROS	Reactive oxygen species
RPMI-1640	Roswell park memorial institute 1640 medium
RQ-PCR	Real-time quantitative polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
SCF	Stem cell factor
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSC	Side scatter
SSP	Single specific primer
TAM	Tumor-associated macrophages
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
TF	Transcription factor
TGF	Transforming growth factor
TLR	Toll like receptor
TNF	Tumor necrosis factor
TRAIL	TNF related apoptosis inducing ligand
TRM	Treatment related mortality
ULBP	UL-16 binding protein
UPS	Ubiquitin proteasome system
VEGF	Vascular endothelial growth factor
WBC	White blood cell count

SYNOPSIS

Background

There has been growing evidence that the immune system plays a major role in the prevention of cancer and in the maintenance of durable remission post chemotherapy. Arsenic trioxide (ATO), as a single agent, is effective in the management of newly diagnosed cases of acute promyelocytic leukemia (APL), still relapse occurs in 5–30% of cases. While there is significant data which address the mechanisms of action of ATO on malignant promyelocytes, limited data is available for its effect on the innate and adaptive immune system. The immune subset recovery post chemotherapy has never been evaluated in APL. Since treatment with an ATO based regimen is the only affordable option for the majority of patients in India, understanding factors that predict clinical outcomes is critical to further improve the efficacy of this therapy. This study evaluates the effects of ATO on the immune response through preclinical studies in mice models and in ongoing clinical trials in patients treated with ATO.

Objectives

1. To study the immune reconstitution in patients with newly diagnosed APL treated with ATO based regimen.
2. To study the cellular and antibody response to PML-RARα oncoprotein in patients with newly diagnosed APL treated with ATO based regimen. To study this response in an APL mouse model as well.

3. To study the effect of arsenic trioxide on the modulation of immune cells.
4. To study the safety and efficacy of PML-RARα targeted DNA vaccine as an adjuvant to ATO in a mouse model of APL.

Hypothesis

1. There is an immune and cellular response to PML-RARα oncoprotein in APL following treatment with ATO. Clinical response and durable remission are dependent on this response.

2. Therapy with ATO enhances both the innate and adaptive immune response against APL cells.

3. PML-RARα targeted DNA plasmid vaccine will further enhance this immune response and synergize with ATO to improve response to therapy.

Methods

Immune reconstitution studies in APL patients: Patients newly diagnosed with APL from March 2010- May 2015 treated with single agent ATO (n= 96) are enrolled in this study after getting written and informed consent (IRB Min no: 7081 dated 17.02.2010). Peripheral blood samples were obtained from patients before treatment , on day 15 after starting ATO, post induction, pre-consolidation, maintenance cycle 2 (6 months from diagnosis), maintenance cycle 6 (10 months from diagnosis) and one year post treatment for immune subset analysis by flow cytometry. Absolute lymphocyte subset counts were calculated for the analysis.

Characterizing Myeloid-derived suppressor cells (MDSC) subsets: Peripheral blood mononuclear cells were evaluated for the presence of immune suppressive MDSCs phenotypically defined as $CD14^{-}HLADR^{-}CD33^{+}CD11b^{+}$ and correlated clinically.

Presence of anti-RARα antibodies in APL patients: The serum collected from APL patients at diagnosis, at maintenance cycle 6 and one-year post treatment were screened for the presence of anti- RARα antibodies by ELISA and was confirmed by immunoblotting.

NK cell cytotoxicity Assay: The cytotoxic activity of NK92MI (NK cell line) against six different myeloid (K562, U937, HL60, UF1, NB4 and NB4EVAsR1) and two lymphoid cell lines (Jurkat E6.1, SUP-B15) was assessed using CFSE/7AAD cytotoxicity assay. In a parallel set of experiments either a leukemic cell line or NK cell line or both were pre-treated with 1μM ATO overnight and cytotoxicity was measured.

Expression profile of ligands and receptors of Natural killer cells: NK92MI cell line were untreated or treated with 1μM ATO and checked for the expression of activating receptors NKp30, NKp44, NKp46, NKG2D and DNAM 1 and inhibitory receptors, KIR2DL1, KIR2DL2, KIR2DS4, KIR3DL1/DL2 and NKG2A by flow cytometry. Similarly, NK ligands CD112, MICA/B, and HLA Class1expression were also analyzed.

NK cell receptor genotyping: The presence or absence of KIR genes were analyzed by PCR using KIR typing kit. Genomic DNA was isolated from bone marrow samples (n=55), and the 15 human KIR genes plus two pseudo genes has been analyzed by this method.

Establishment of the preclinical animal model of APL treated by arsenic trioxide: The transplantable APL mice model was established by injecting 6 week old wild type FVB/N mice with leukemic blasts (5x 10^4 cells) intravenously obtained from the laboratory in France (a collaborative work with Dr. Christine Chomienne and group, INSERM, Paris) after the approval from animal ethics committee (IAEC 2/2012). The experimental groups were: a) Placebo, b) ATO (5 µg/g mice for 28 days i.p), c) ATRA (all-trans retinoic acid 5mg subcutaneous implantation of 21day release pellet), d) ATO and ATRA and e) ATO+ATRA+DNA vaccine (100µg was given in 3 doses intramuscularly). Mice were monitored for blood counts, organ pathology, survival and other parameters.

Cellular therapy by NK and IL-15 in APL transplantable mouse model: FVB/N mice treated with ATO or ATRA or in combination was given three doses of $5x10^5$ NK cells (sorted from FVBN spleen) with or without IL-15(100ng /mouse given in 5 doses).

Major findings

Analysis of lymphocyte subset reconstitution patterns has shown that all the subsets were below the normal range at diagnosis except the dendritic cell subset, and NKT subset lies in the near normal ranges. Following treatment, there was a differential pattern of immune reconstitution in different lymphocyte subsets. The earliest recovery to the normal range was seen in the CD8 subset while the mean CD4 subset reached the normal range only by pre-consolidation time point. The CD4/CD8 ratio remains inverted until the start of consolidation therapy. There was a significant delay in the

reconstitution of NK cells ($CD56^{+}CD3^{-}$) where it has not recovered to normal levels till the end of one year post chemotherapy and the median absolute counts of $CD56^{dim}$ subset were lower throughout the therapy when compared with $CD56^{bright}$ subset.

The median percentage of MDSC in APL (n=70) was 0.63% (range: 0.04-5.94) and of HC (n=10) was 0.7% (range: 0.11-0.97).In AML (n=137) the median MDSC was 1.07 % (range: 0.01-31.1%) and there is a significant heterogeneity among the AML cases. There was no significant association among cases with APL with conventional risk factors.

In order to further evaluate the mechanism to explain the delayed recovery of NK subset post treatment with ATO**,** we undertook a series of experiments to address the impact of ATO on NK cells. We observed that ATO has no direct cytotoxic effect on NK92MI cell line (IC50-3.8µM) and on its proliferation but the exposure of NK92MI cell line to 1µM ATO (sub-lethal doses) for 6 hours resulted in increased expression of activating receptors NKG2D, NKP30 and KIR2DS4 and inhibitory receptor NKG2A and decrease in inhibitory receptors KIR3DL1/DL2.There was a significant increase in activating ligand MICA/B when NB4 cells were exposed at 1µM ATO for 6 hours (n=3; P=0.016) which was not significant in any of the other cell lines. Similar significant increased expression of CD112/Nectin-2 (DNAM-1 ligand) and HLA Class I was also seen. We observed that NB4 when treated overnight with 1µM ATO significantly increased the cytotoxic effect of NK92MI cell line at all effector: target ratios evaluated (n=5; P=0.0023). No other cell line showed a similar increase in cytotoxic effect

following exposure to ATO at these concentrations. We also observed a delay in the differentiation of NK from CD34 cells on exposure to ATO which correlated with the low expression of NK transcription factors involved in NK maturation. This also explains the low $CD56^{dim}$ population in APL patients throughout the therapy. Out of the 55 patients screened for KIR genotyping, 31% of patients have A haplotype and 69% with B haplotype, and there was no association with any specific genotype or haplotype with the risk of relapse or any other clinical outcome parameters but there was a trend towards significance of inhibitory receptor KIR2DL2 in relapse patients.

We were able to reproduce the observation of synergy between ATRA and the DNA plasmid vaccine in the APL mouse model, but the use of DNA vaccine with ATO did not contribute any additional benefit in survival. ATO+ATRA+DNA and ATO+ATRA show a similar significant increase in survival than the other groups. Also, we looked at the effect of infusion of NK cells along with ATO in APL mouse model and also the effect of IL-15 as it is known to activate NK cells. ATO-treated mice with NK cells, and IL-15 showed prolonged survival when compared with ATO alone. We have also seen the presence of RARα antibodies in FVB/N long-term survivors treated with ATO+ATRA+DNA and in APL patients one-year post treatment with ATO.

Significance of the findings

This study demonstrated that there is significant heterogeneity in the pattern of immune reconstitution in different lymphocyte subsets post treatment of APL with ATO.

Modulation of immune recovery and the presence of RARα antibodies could potentially improve leukemia clearance and maintenance of remission. The delay in the NK cell reconstitution pattern in APL patients treated with single agent ATO was observed for the first time. We also observed that exposure to sub lethal doses of ATO up-regulates NK ligands and receptors and enhances NK-mediated cytolytic activity. The mechanism to explain the delayed recovery of NK subset post treatment with ATO needs further evaluation. However, the delayed NK cell recovery raises the potential possibility of using NK cell therapy to enhance the effect of ATO in the treatment of patients as we observed from the cell line data. Also, the observations from APL mouse model have significant clinical implications in using NK cells to enhance the efficacy of ATO. The DNA plasmid-based vaccine strategy evaluated in APL mouse model requires further evaluation. We anticipate that with our findings, augmenting therapeutic interventions in the high-risk subset of patients by either adding chemotherapy or the immune response by a vaccine based strategy can be promising for improved clinical outcomes in patients with acute promyelocytic leukemia.

INTRODUCTION

The first evidence for the ‘existence of a particular type of acute myelogenous leukemia with a very rapid deadly course of only a few weeks duration, severe bleeding tendency and white blood cell (WBC) picture dominated by promyelocytes, traces back to 1957 when a Norwegian hematologist, L.K Hillestad, described acute promyelocytic leukemia (APL) as a distinct entity (Hillestad, 1957). APL is a subtype of acute myeloid leukemia (AML) characterized with distinct molecular and clinical features and majority of cases characterized by the fusion between retinoic acid receptor alpha gene (*RARα*) on chromosome 17 and the partner gene promyelocytic leukemia (*PML*) on chromosome 15 as a result of reciprocal translocation t(15;17)(q24;q21), leading to the expression of a novel PML-RARα oncoprotein (Sirulnik et al., 2003). This chimeric protein, a unifying signature of APL, blocks myeloid differentiation, which results in the accumulation of abnormal promyelocytes in the bone marrow. Even though relatively rare among hematopoietic malignancies (approximately 10% of AML cases), APL is biologically, clinically and prognostically different from other subtypes of AML. APL associated with a distinct coagulopathy results in disseminated intravascular coagulation (DIC), contributing to significant morbidity and mortality.

Early advances in the treatment of APL with conventional anthracycline-based chemotherapy from the 1970s were associated with high treatment-related mortality (TRM), and as a result, it remained a subset of AML with the worst prognosis and the most challenging to manage (Bernard et al., 1973). The first breakthrough was the

introduction of all-trans retinoic acid (ATRA) in the late 1980s, a physiologically active derivative of Vitamin A, in the management of APL. Treatment of leukemic blasts with pharmacological doses of ATRA results in terminal differentiation and importantly reduced early treatment-related mortality due to coagulopathy (Huang et al., 1988, Castaigne et al., 1990, Fenaux et al., 1993). The combination therapy with ATRA and anthracyclines in induction has lead to a dramatic improvement in event-free survival (EFS) and overall survival (OS) (Wang, 2003). With currently used ATRA combined with chemotherapy regimens, 70-80% of patients are alive and free of disease at five years (Lowenberg et al., 2003). For this reason, APL has become the paradigm of differentiation therapy in the field of medicine.

Arsenic trioxide (ATO) has been successfully introduced both as a single agent and in combination with conventional agents in the late 1990s in the treatment of newly diagnosed APL, (Shen et al., 1997, Soignet et al., 1998, Mathews et al., 2002). Arsenic trioxide, in a dose-dependent manner, exerts its therapeutic effect by promoting degradation of the oncoprotein that drives the growth of APL cells (Chen et al., 1997). ATO, as a single agent, is effective in inducing molecular remission in patients with relapsed APL and equally effective in newly diagnosed cases of APL. Many studies have demonstrated the ability of this agent when used either as a single agent or in combination with ATRA, and other chemotherapeutic agents which can induce durable remissions with minimal toxicity in newly diagnosed patients. In low-risk patients, defined based on the WBC count at diagnosis, this agent is very effective while among

high-risk cases the clinical outcome is inferior to conventional therapy (Mathews et al., 2010). Thus APL, once considered as the most malignant human leukemia with worst prognosis, has evolved in the past few decades into a highly curable disease.

Despite its efficacy in the treatment of APL, it has been noted that 10–20% of newly diagnosed patients mostly within the high-risk subset will relapse, after treatment with ATO-based regimens (Tallman, 2007, Mathews et al., 2010). There is limited data regarding the best consolidation regimen for patients with relapsed APL who have achieved molecular remission with an ATO based regimen. In the absence of consolidation with autologous stem cell transplantation, there is a high incidence of relapse (30-50%) (Thirugnanam et al., 2009). Recently it was recognized that the mutations in the ATO binding B2 domain of PML-RARα oncoprotein could result in a lack of responsiveness to ATO, but there is limited data available on the frequency of these mutations in relapsed patients treated with ATO (Zhang et al., 2010, Goto et al., 2011). Due to the lack of financial support and the medical system that exists in India where the individual pays health care expenses, the majority of patients with relapsed APL cannot afford stem cell transplantation.

Interventions that could improve the clinical outcome of such patients could have significant implications for us and in other developing countries. There has been growing evidence that the immune system plays a role in the prevention of cancer and also in the maintenance of durable remission post chemotherapy. Some of the anticancer drugs used in the clinic can suppress the patient immune system, and some of them can

elicit an immune response to completely eradicate the leukemic cells (Kepp et al., 2011). Data from solid tumors demonstrate the benefit of conventional treatment to decrease the tumor-induced immune suppressive cells such as Tregs and myeloid-derived suppressor cells and their significance in prognosis (Nadal et al., 2007, Giallongo et al., 2014). Various agents were evaluated for their ability to induce the natural killer cell (NK) activating ligands on leukemic cells to augment the NK-mediated anti-leukemic effect. Histone deacetylase inhibitor, sodium valproate, and all-trans-retinoic acid were shown to induce NK ligand expression in certain types of malignancies including AML (Diermayr et al., 2008). The proteasome inhibitor bortezomib and histone deacetylase inhibitors were also shown to improve NK-cell-mediated lysis of AML cells through TNF-related apoptosis-inducing ligand (TRAIL) or the death-receptor pathway (Hallett et al., 2008).

It is believed that the immune response or re-activation play a significant role in preventing relapse. In the context of APL, it has been demonstrated that an adaptive immune response is necessary for sustaining long-term molecular remission in a transplantable mouse model of APL (Westervelt et al., 2002). It has also been recognized that, in mice and patients with APL, antibodies against RARα and myeloid proteins are frequently present and potentially contribute to the maintenance of sustained molecular remission (Robin et al., 2006). Another study had shown an association with the presence of HLA B13 allele and the risk of relapse among patients with APL treated with ATRA and chemotherapy again indirectly stressing the importance of an immune

response in sustaining the response of therapy (Bolognesi et al., 2000). Few studies have shown the immunomodulatory property of ATO in up-regulating the NK ligands on tumor cells thereby increasing the susceptibility of cancer cells against NK cells (Kim et al., 2008, Poggi et al., 2009). Recently studies have shown the role of Killer Immunoglobulin-like receptor (KIR) gene haplotype as a predictor of disease outcome (Stringaris et al., 2016). Various studies have looked at immunotherapeutic strategies such as upregulation of functional activating receptors on NK cells using interleukin 15 (IL-15) to improve NK cell cytotoxicity (Boyiadzis et al., 2008).

Additional strategies have been attempted to study the role of immune response in APL using an APL mouse model. PML-RARα targeted DNA vaccine or a non-specific DNA vaccine, pVAX14 Flipper (pVAX14) can induce protective immunity and can synergize with ATRA and ATO to produce durable remissions (Padua et al., 2003, Le Pogam et al., 2015, Patel et al., 2015). An adjuvant effect of ATRA on the immune responses may also contribute to the efficacy of this agent in inducing and sustaining molecular remission beyond that of its known cellular effects of differentiation in APL (Furugaki et al., 2010). The available data suggests that the immune response co-operates with therapy to ensure cure in this malignancy and this needs to be studied systematically. A better understanding of the mediators, regulators, and mechanisms to augment such immune responses could have potential clinical application in reducing the risk of disease recurrence. Since treatment with an ATO based regimen is the least expensive and accessible option for the majority of patients in India, understanding

factors that predict clinical outcomes is critical in improving the efficacy of this therapy further.

1.1 Rationale and Hypothesis

At our center, we have been treating acute promyelocytic leukemia with a single agent ATO based regimen since 1998. We have reported our short and long term findings with this regimen (Mathews et al., 2002, Mathews et al., 2006, Mathews et al., 2010). This regimen is especially relevant to us since it offers a cost-effective regimen and gives us the ability to treat almost all patients diagnosed at our center. In addition to their direct effect on the malignant cells which contributes to their efficacy, the role in enhancing immune-mediated response needs a better understanding. Unlike data that is available after therapy with ATRA, there is limited data on the immune response after treatment of APL with ATO. The role of ATO towards innate and adaptive immune system and the immune response over time needs to be addressed. So we hypothesized that

1. There is an immune and cellular response to PML-RARα oncoprotein in APL following treatment with ATO. Clinical and durable remission are dependent on this response.

2. Therapy with ATO enhances both the innate and adaptive immune response against APL cells.

3. PML-RARα targeted DNA plasmid vaccine will further augment this immune response and synergize with ATO to improve response to therapy.

We had taken the benefit of our expertise in the immunomonitoring of APL mice models and the access to APL patients to address the role of the immune response which may have an impact on long-term relapse-free survival, immunosuppression or antibody induction. In this study, we attempted to evaluate the effects of ATO on the immune response through preclinical studies in mice models and ongoing clinical trials in patients treated with ATO. Towards this we tried to investigate the effect of ATO on the immune response to RARα, its impact on cellular subset reconstitution and immunomodulation in mice and patients. The DNA vaccine approach in mice model provided additional details in our study. By augmenting the immune responses to this disease, we hope that it is possible to improve clinical responses further with ATO.

1.2 Objectives of the study

1. To study the immune reconstitution in patients with newly diagnosed APL treated with ATO based regimen.

2. To study the cellular and antibody response to PML-RARα oncoprotein in patients with newly diagnosed APL treated with ATO based regimen. To study this response in an APL mouse model as well.

3. To study the effect of arsenic trioxide on the modulation of immune cells in APL patients and APL cell lines.

4. To study the safety and efficacy of PML-RARα targeted DNA vaccine as an adjuvant to ATO in a mouse model of APL.

1.3 Brief overview of the thesis chapters

1.3.1. Literature review

Following the introduction, hypothesis and objectives of the current study, the next major chapter in this thesis is the literature review, which elaborates on the disease model in this study, its pathologic and molecular mechanisms and the standard of care. The central focus was on immune response, immunomodulation and ways of immune escape in leukemia. The role of chemotherapeutic agents in enhancing the immune response was described in this section. A mouse model developed to study the disease has been described in detail towards the last part.

1.3.2. Materials and Methods

Different strategies for evaluating the immune and cellular response was described in detail in the methodology section. The study design was outlined and the various techniques and methods used for conducting experiments and analyzing data were described. The methodology section was broadly divided into two parts, the first part gives a detailed description of the experiments to address the role of cellular and immune response in patients with APL treated with ATO and in cell lines. The experiments to assess the role of natural killer cells and immunomodulation of ATO has

been studied. The second part of methodology deals with the establishment of preclinical APL mouse model and interventions done with this model.

1.3.3. Results

The immune reconstitution of cellular subsets post treatment with ATO forms the first major part of the results. The role of ATO on modulating the immune cells has been evaluated in the next part giving emphasis to the role of natural killer cells. Enhancing the cytolytic activity of NK cells and the alteration of NK ligands and receptors by ATO has been investigated. The presence of anti-RARα antibodies in APL patients and in an APL mouse model was also assessed. The last major part involves evaluating the efficacy of DNA vaccine as an adjuvant to ATO in an APL transplantable mouse model and the role of NK cell therapy using this model.

1.3.4. Discussion

The significance of the major results obtained in this study has been described in detail in this section. The results obtained in this study have been discussed with the existing scientific data with implications to the clinic.

1.3.5. Summary and Conclusion

The key observations from this doctoral work have been summarized in this chapter and the scope to take up this work forward has been suggested. The references cited in the text were listed in Bibliography.

REVIEW OF LITERATURE

'The need to deal with pathogens and foreign bodies has driven the evolution of the vertebrate immune system, so it should not be surprising that experiments with infectious agents have often illuminated key elements of the underlying mechanisms'

- *Peter C. Doherty, Nobel Lecture, December 8, 1996*

'Immunity can be life-long if we get it right.' The evolution by natural selection and enormous variations since the origin of life has favored the formation of a complex evolutionary unit, the immune system which takes up the difficult challenge of defending against a diversity of foreign pathogens, while simultaneously maintaining tolerance towards self-antigens which is a paradigm shift in immunology. Paul Ehrlich and Elie Metchnikoff (1908 Nobel Prize in Physiology or Medicine) were the two eminent scientists in this field who brings about divergent views of how the body protects itself against foreign invaders. These cellular elements are derived ultimately from the same progenitor cells - the hematopoietic stem cells.

2.1. Hematopoiesis

Production and sustaining of blood cells throughout one's life is the property of hematopoietic stem cells (HSC) that resides as rare cells in the bone marrow. This process, known as hematopoiesis, conventionally a hierarchical system, yield blood precursors devoted to unilineage differentiation and production of mature blood cells,

including red blood cells, megakaryocytes, myeloid cells (monocyte/macrophage and neutrophil), and lymphocytes (Orkin, 2000). These cell types function as protection against infections, oxygen transport and maintaining hemostasis. HSCs are capable of self-renewal, they can generate more HSCs and have the potential to differentiate into various progenitor cells that eventually commit to further maturation along specific pathways to all blood cell lineages. HSCs comprise only 0.01–0.2% of total bone marrow (BM) mononuclear cells in humans (Pang et al., 2011). The repertoire of cell-surface molecules enables HSC purification and their detailed functional characterization (McKinney-Freeman et al., 2009).

Within the bone marrow, both the endosteal (cortical and trabecular regions) and perivascular microenvironments have been described as stem cell niches that support the HSCs. Quiescent or slow-cycling long-term hematopoietic stem cells (LT-HSCs) localize close to the bone and bone marrow interface, a site known as the endosteal or osteoblastic niche and the fast-cycling short-term HSCs (ST-HSCs) may be found in close proximity to sinusoidal endothelial cells and perivascular cells. This site, which is known as the perivascular niche, supports the proliferation and differentiation of HSCs (Balduino et al., 2005).

The multipotent progenitors (MPPs) arises from the ST-HSCs lose self-renewal potential and differentiate into all multilineages. MPPs further give rise to oligopotent progenitors which are common lymphoid and myeloid progenitors (CLPs and CMPs). CMPs advance to megakaryocyte/erythrocyte progenitors (MEPs), granulocyte /

macrophage progenitors (GMPs) and DC progenitors. CLPs give rise to T cell progenitors, B cell progenitors, NK cell progenitors and DC progenitors (Orkin, 2000). Ultimately, these cells give rise to B cells, NK cells, T cells, granulocytes, monocytes, erythrocytes and megakaryocytes (Figure 1).

Hematopoietic cells of the body are produced and destroyed under precise control of many different biological response modifiers, including the colony-stimulating factors, interleukins, and interferon. Extensive studies have shown that hematopoiesis is guided by cell-specific transcriptional regulators and associated chromatin factors that function to establish all mature blood cells for stem cell production, self-renewal, and survival (Orkin and Zon, 2008). The pivotal role of microRNAs (miRNAs), a class of short non-coding RNAs, in fine-tuning the hematopoietic system has also emerged in the past several years (Chen et al., 2004). Recent studies from Scadden and his group investigated the roles of bone marrow endothelial cells (BMECs) as regulators of hematopoiesis (Itkin et al., 2016). Thus self-renewal and differentiation of HSCs are tightly regulated, and various hematopoietic stress results in its extensive proliferation. Understanding the cellular biology of HSCs is, therefore, elementary for any clinical or basic research in immunology and especially in leukemia.

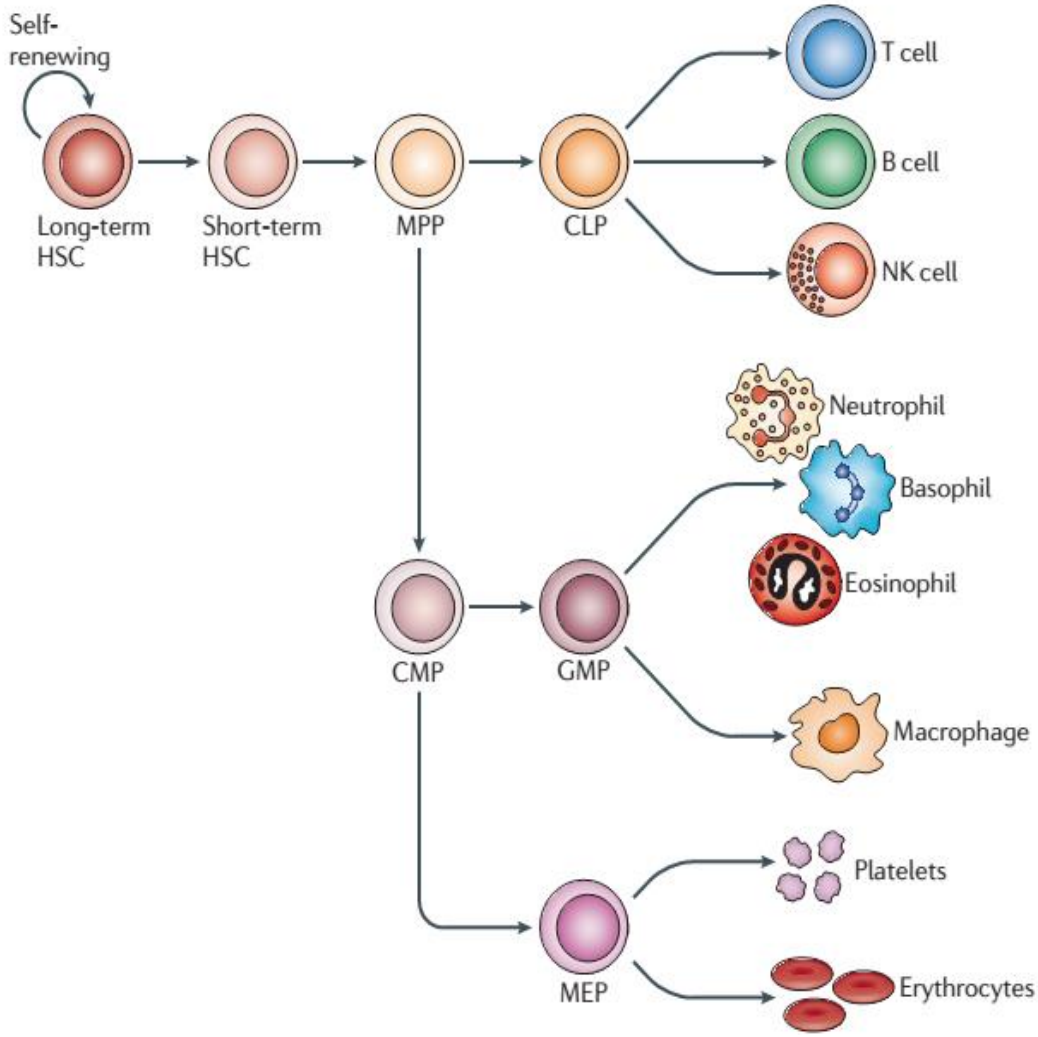


Figure 1. Hierarchical organization of the hematopoietic system. *Macrophages, eosinophils, neutrophils, basophils, and NK cells are parts of the innate immune system. T and B cells are part of the adaptive immune system. Erythrocytes are red blood cells, and megakaryocytes produce platelets for clotting. Adapted from (King and Goodell, 2011).*

2.2. The Immune system

The mammalian immune system is equipped with an extremely sophisticated defensive system which is a complex of biochemical processes enabling efficient detection and deletion of pathogens that threaten host viability. This incredible complex microscopic army, are the product of millions of years of evolution is held up by a complex network of organs, tissues and cells. The main function of this irreplaceable immune cells is to monitor tissue homeostasis, to protect against invading pathogens and to eliminate damaged cells and also implicated in the pathogenesis of many chronic diseases (Delves and Roitt, 2000).

Based on antigen specificity and timing of activation, the immune system is composed of two distinct compartments - innate and adaptive. Innate and adaptive immunity act as a complementary network of self-defense against foreign threats. For the accomplishments in understanding and unifying the evolutionarily ancient innate immune response and modern adaptive immunity Jules Hoffmann, Bruce Beutler and the late Ralph Steinman shared The Nobel Prize in Physiology or Medicine in 2011.

2.2.1. Innate immune system

Innate immunity forms the first line of defense against foreign pathogens after it has overcome the physical and chemical barriers. It is evolutionarily conserved among all multicellular organisms. Innate immunity constitutes the antigen-independent immune mechanism involving surface barriers including antimicrobial peptides,

mononuclear phagocytes (monocytes and macrophages), polymorphonuclear cells (neutrophil, eosinophil and basophil), natural killer (NK) cells, dendritic cells (DCs) and complement activation (Medzhitov and Janeway, 2000). The molecular components of innate responses also include complement, acute-phase proteins, and cytokines such as the interferons (Delves and Roitt, 2000).

Monocytes are rapidly recruited to the tissue upon tissue damage or infection, where they can differentiate into tissue macrophages or dendritic cells. (Gregory and Devitt, 2004). Macrophages are professional phagocytes of apoptotic cells. DCs are professional antigen-presenting cells (APC) and by phagocytosis, endocytosis, pinocytosis or receptor-mediated uptake, DCs capture antigens for immune presentation (Liu et al., 2001). NK cells are lymphocytes capable of both directly killing of target cells and production of immunoregulatory cytokines. NK cells and DCs are especially important in tumor immunology and immunotherapy.

In innate immunity, signaling is through germ-line receptors (pattern recognition receptors, PRRs) that recognize the pattern of exogenous agents and antigens (pathogen-associated molecular patterns, PAMPs) which include scavenge receptors and toll-like receptors (TLRs). These receptors are primarily present on monocytes/macrophages and dendritic cells, which may be activated as antigen-presenting cells (Takeuchi and Akira, 2010). They may also be present on natural killer (NK) cells, endothelial cells, and cells of the adaptive immune system. The principle function of PRRs includes activation of complement, opsonization, phagocytosis, induction of inflammatory cytokines and

induction of programmed cell death (apoptosis). Thus the innate arm of immunity is composed of those immunological effectors that provide robust, immediate, and non-specific immune responses.

2.2.2. Adaptive Immune system

The adaptive system consists of two broad sets of antigen-responsive cells, the B and T lymphocytes. B lymphocytes (humoral immunity), $CD4^+$ helper T lymphocytes and $CD8^+$ cytotoxic T lymphocytes (CTLs) (cell-mediated), distinguish themselves from innate immunity by expression of somatically generated, diverse antigen-specific receptors, which are formed as a consequence of random gene rearrangements and allow a flexible and broader repertoire of responses than innate immune cells. The adaptive immune system ensures long-lasting immunological memory against re-infection. The extend of innate (natural) response is same even after the repeated encounter with the pathogen, whereas acquired (adaptive) responses improve on repeated exposure to infection (Delves and Roitt, 2000).

Acquired responses involve the proliferation of antigen-specific B and T cells, which occurs when the surface receptors of these cells bind to the antigen. Antigen-presenting cells, present the antigen to lymphocytes and collaborate with them in response to the antigen. B cells secrete immunoglobulin, the antigen-specific antibodies responsible for eliminating extracellular microorganisms. T cells help B cells to make antibody and can also eradicate intracellular pathogens by activating macrophages.

Innate and acquired responses work together to eliminate pathogens thus increasing the efficiency of immune responsiveness (Figure 2).

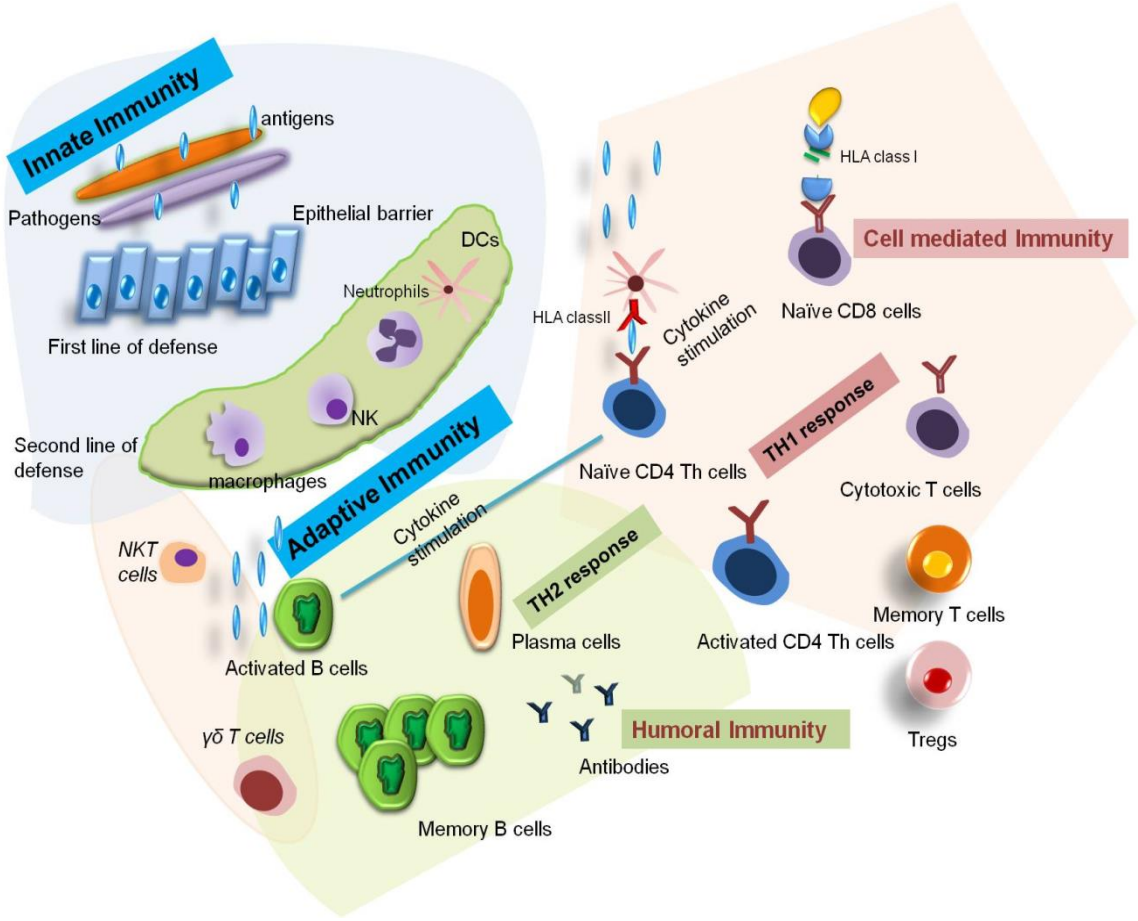


Figure 2. The innate and adaptive immune system. *The innate immune response functions as the first line of defense against infection. It consists of granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells and natural killer cells and soluble factors. The adaptive immune response is slower to develop but manifests as increased antigenic specificity and memory. It consists of antibodies, B cells, and $CD4^+$ and $CD8^+$ T lymphocytes. Natural killer T cells and γδ T cells are cytotoxic lymphocytes that form the interface of innate and adaptive immunity. Th1 cells evoke cell-mediated immunity and Th2 cells evoke strong antibody response.*

2.2.3. Antigen processing and presentation

The proteins and the peptides are handled by the professional antigen presenting cells (APCs) including macrophages and dendritic cells through different cellular pathways for the generation of an effective and specific immune response. T cell recognition of APCs depends on the peptides bound to major histocompatibility complex class I (MHC-I) and class II (MHC-II) molecules. MHC Class I molecules present endogenous antigenic peptides generated by proteasomal proteolysis to $CD8^+$ T lymphocytes, and MHC-II molecules bind to exogenously synthesized peptides generated by lysosomal proteolysis in the endocytic and phagocytic pathway and present to $CD4^+$ T helper cells (Jensen, 2007). Another phenomenon called cross-presentation were exogenous antigens can be presented by MHC class I molecules, and endogenous antigens can also be presented by MHC class II. DCs mediate this process which initiates a primary response by naive $CD8^+$T cells which is termed as cross-priming (Joffre et al., 2012).

2.3. Tumor Immunology

2.3.1. Hallmarks of Cancer

A normal cell acquires genetic changes and mutations over time and slowly transforms into a precancerous state and ultimately into cancer is collectively defined as the hallmarks of cancer. In addition to malignancy, cancer is a disease affecting microenvironment and immunity. Douglas Hanahan of the University of California, San Francisco and Robert Weinberg of the Massachusetts Institute of Technology described the Hallmarks of Cancer elaborately in two landmark publications in Cell in 2000 and then in 2011.

As defined by them, the tumorigenic process initiates from six acquired capabilities: self-sufficiency in growth signals, resistance to growth-inhibitory signals, resistance to apoptosis, limitless growth potential, sustained angiogenesis and potential to metastasis that allows cancer cells to survive, proliferate and disseminate (Hanahan and Weinberg, 2000). The above functions are made possible by two characteristics, genomic instability of cancer cells and inflammatory state of malignant cells. Avoidance of immunosurveillance, a seventh potential hallmark of cancer, resulting in the escape of tumor from anticancer immune responses, has been introduced recently along with the other emerging hallmark deregulation of cellular energetics (Dunn et al., 2004, Hanahan and Weinberg, 2011). The major hallmarks of cancer are depicted in Figure 3.

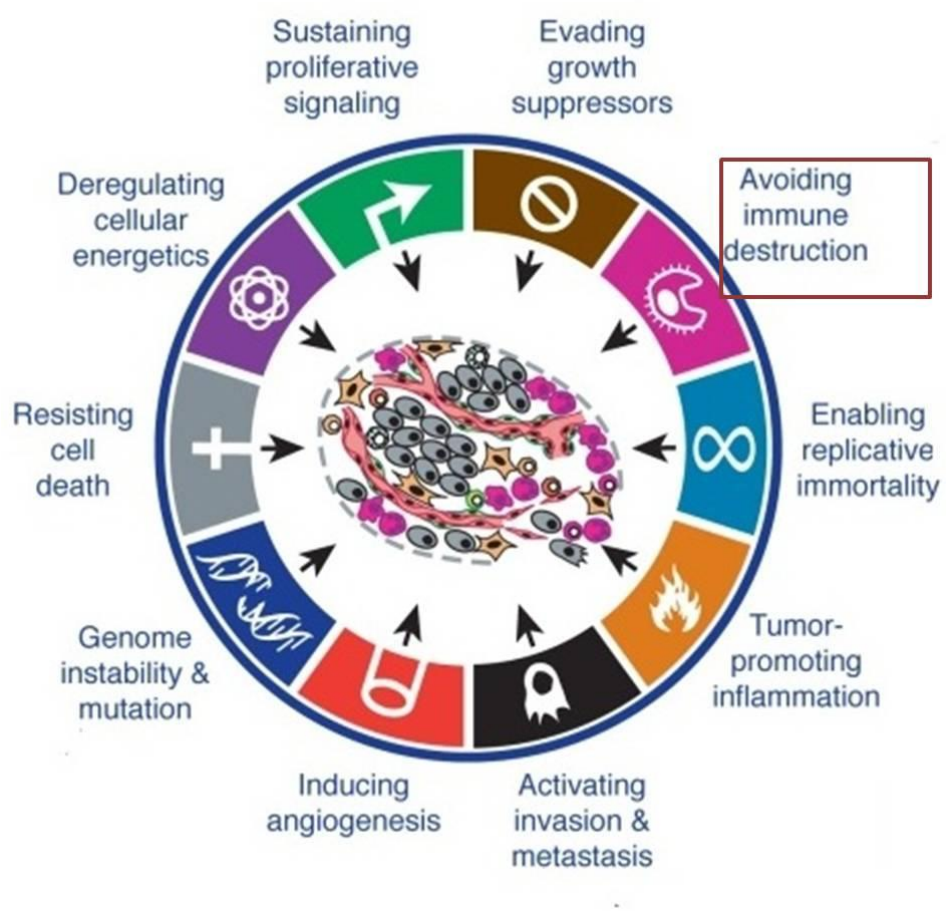


Figure 3. Hallmarks of Cancer. *Cancer cells are considered as altered self that has escaped from the normal growth regulating mechanisms. The main acquired capabilities are self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, tissue evasion, and metastasis and the enabling characteristics are genomic instability and inflammation. The emerging hallmarks were avoiding immune destruction and deregulation of cellular energetics. Adapted from (Hanahan and Weinberg, 2011).*

There is an increased evidence of the role of immune system in evading pathogens, inflammation and in cancer biology. There are a number of mechanisms by which the immune system can recognize malignant cells and similarly mechanisms by which the tumor can evade the immune system (Dunn et al., 2002). Cells of both innate and adaptive arms of the immune system can mediate antitumor immunity. The immune system can respond to tumors in three ways: timely elimination or suppression of viral infections and protecting the host from virus-induced tumors, elimination of pathogens and preventing the establishment of an inflammatory environment favorable for tumorogenesis and by reacting against tumor-specific antigens (molecules that are unique to cancer cells) or against tumor-associated antigens (molecules that are expressed differently by cancer cells and normal cells) (Finn, 2008).

Thomas and Burnet in the 1970s proposed that lymphocytes continually identify and eliminate newly arising cancer cells in immunocompetent hosts called immune surveillance (Burnet, 1970). The concept of immune surveillance opened up a new insight into the role of immune system, and its interaction with malignant cells in which either the tumor is modified by the immune system or the immune cells are modified by the tumor is termed as Immune editing. Cancer immunoediting is a complex extrinsic tumor suppression process proceeds sequentially through three distinct phases or Es ‘elimination’, ‘equilibrium’and ‘escape’ which takes place when the intrinsic tumor suppressor mechanisms have failed (Dunn et al., 2004) (Figure 4).

2.3.2. Cancer Immunoediting

Elimination: Innate and adaptive immune systems work together to detect the presence of a developing tumor and eliminate them before it becomes clinically apparent. The danger signals like cytokines (Type1 IFNs), damage-associated molecular pattern molecules (DAMPs), stress ligands (MICA/B) expressed on the surface of tumor cells were identified by the immune cells, leading to release of pro-inflammatory and immunomodulatory cytokines. This establishes a microenvironment that facilitates the development of a tumor-specific immune response.

Equilibrium: When a cancer cell variant survives the elimination phase, it enters the equilibrium phase, the longest phase of the cancer immunoediting process which extends throughout the life. The cell outgrowth is prevented by immunologic mechanisms and T cells, IL-12, and IFN-γ are required to maintain tumor cells in a state of functional dormancy. Editing of tumor immunogenicity occurs in the equilibrium phase.

Escape: In this phase due to the constant immune selection pressure placed on genetically unstable tumor cells, they are no longer recognized by adaptive immunity due to poor immunogenicity (antigen loss and defects in antigen processing or presentation) and become insensitive to immune effector mechanisms and finally induce an immunosuppressive state within the tumor microenvironment. The tumor outgrowth is no longer blocked by immunity and they emerge to cause clinically apparent disease (Schreiber et al., 2011).

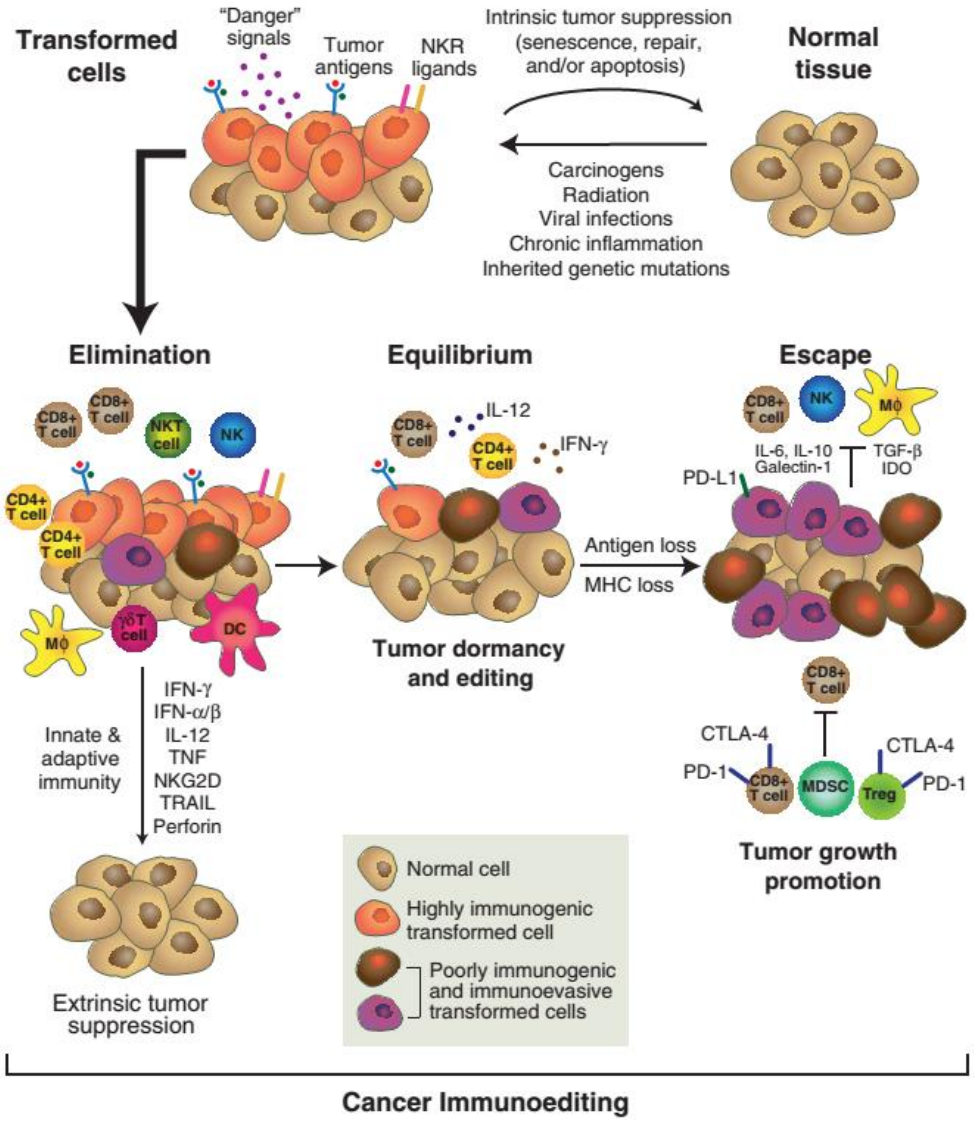


Figure 4.The concept of cancer immunoediting. *When nascent transformed cells emerge, they were easily eradicated by innate and adaptive immune responses in the elimination phase. If a rare cancer cell variant is not destroyed in the elimination phase that has low immunogenicity, it may then enter the equilibrium phase, which its outgrowth is prevented by immunologic mechanisms. However as a consequence of constant immune selection pressure these tumor cells are no longer recognized by adaptive immunity, become insensitive to immune effector mechanisms and results in tumor progression (Schreiber et al., 2011).*

2.3.3. Cells involved in pro-tumor and anti-tumor responses

Cells of antitumor responses

Immune-mediated eradication of neoplastic cells requires induction of a strong, long-lasting antitumor response. There is increasing evidence to suggest that some patients with cancer can elicit an antitumor immune response that has the potential to control or eliminate cancer (Disis, 2010). The cellular machinery of a tumor includes infiltrating lymphocytes, NK cells, macrophages, DCs, eosinophils, mast cells, and immature myeloid cells.

IFN-γ producing $CD4^+$ Th1 cells and $CD8^+$ T cells play a major role in inhibiting and killing tumor cells. Th1 cells secrete cytokines like IFNγ, TNFα, and IL2 which helps CTLs for tissue destruction via an MHC-restricted and perforin-dependent manner. Dendritic cells stimulate Th1 cells, and IFN-producing killer dendritic cells (IKDCs) elicit an antitumor adaptive response in a TRAIL and perforin-dependent manner (Malmberg and Ljunggren, 2006). Also activated $CD4^+$T cells recognize tumor-infiltrating macrophages in an MHC-class-II-dependent manner which results in converting interleukin-10 (IL-10)-producing M1 macrophages into IFNγ-producing M2 macrophages. In the initial stages of tumor development, Type 1 macrophages (M1) infiltrate and release pro-inflammatory cytokines and chemokines, such as CXCL19 and CXCL10, and activate Th1, Th17 and NK cell development and differentiation (Zamarron and Chen, 2011). NK cells recognize and kill the MHC class I-deficient

tumor cells through the balance between activating and inhibitory receptors (Caligiuri, 2008).

Cells promoting tumor development

Regulatory T cells (Treg cells) and Myeloid-derived suppressor cells (MDSCs) are two major immunosuppressive cells. The secretion of TGFβ and IL-10 by Tregs in tumors create an immunosuppressive environment that masks antitumor effector responses by $CD4^+$, $CD8^+$ and NK cells (Kerkar and Restifo, 2012). Tumor-derived factors recruit MDSCs and prevent their differentiation into mature dendritic cells and inhibit tumor-specific T cells by overproduction of nitric oxide and have increased arginase-1 activity. Tumor-associated macrophages (TAMs) which belong to the M2 class of macrophages produce arginase-1, IL-10, TGFβ and prostaglandin E2 (PGE2) favor TH2-cell responses (Gabrilovich and Nagaraj, 2009).

Plasmacytoid DCs (pDCs) activated by interleukin-3 (IL-3) and CD40 ligand (CD40L) also promote the differentiation of naive $CD4^+$ and $CD8^+$T cells into Th2 cells producing immunosuppressive cytokines such as vascular endothelial growth factor (VEGF), TGFβ, galectin or indoleamine 2,3 dioxygenase (IDO) (Zitvogel et al., 2006). Downregulation or mutation of death receptors, mutations of caspase-8 and overexpression of FLIP (caspase-8 (FLICE)-like inhibitory protein) or decoy receptors for TRAIL also inhibits CTL-induced killing (Igney and Krammer, 2002). IL-13 from NKT cells activate myeloid suppressor cells to produce TGFβ can also suppress CTL

activity. The negative regulatory receptors on T cells or the immune checkpoints like cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed death-1 (PD-1) engage with the B7 ligands diminishes APC function and costimulation (Disis, 2010). The cells involved in pro-tumor and anti-tumor immunity is depicted in Figure 5.

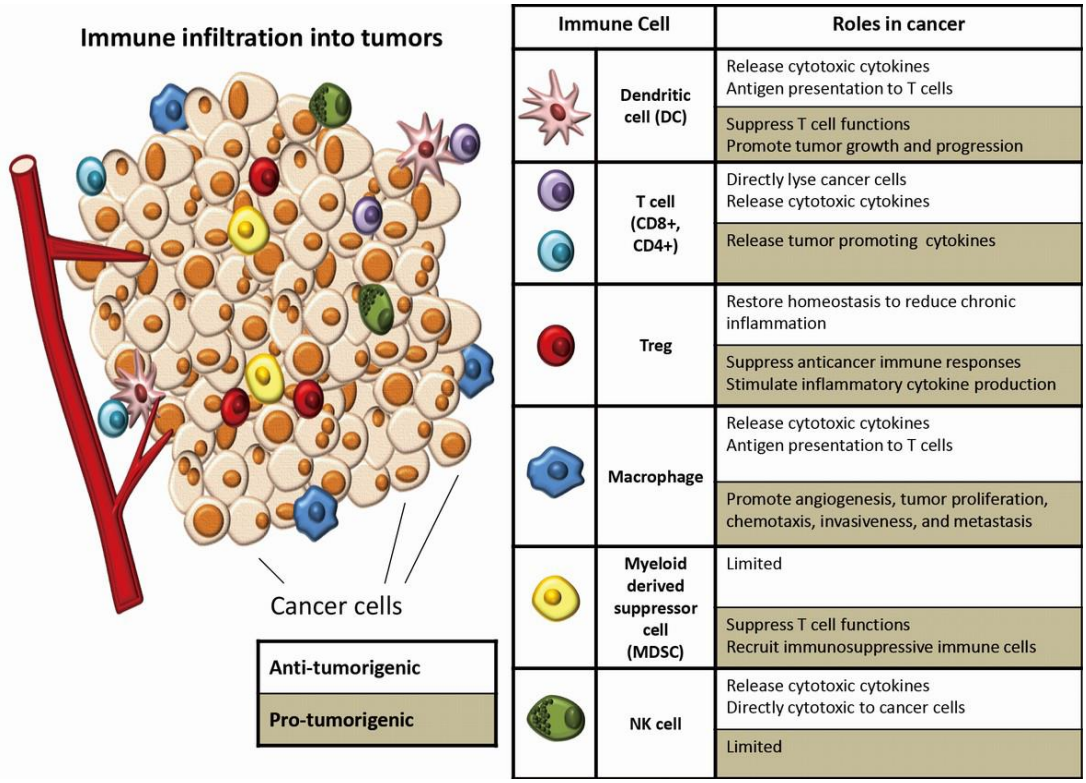


Figure 5. Immune cells in the tumor immune microenvironment*. Adapted from (Markman and Shiao, 2015)*

2.3.4. Immune escape mechanisms in cancer

There are numerous ways by which tumor cells can paralyze immunosurveillance. There are three major mechanisms of immune escape. (i) escape by the loss of recognition. (ii) escape by the loss of susceptibility and (iii) escape by

induction of immune suppression (Malmberg and Ljunggren, 2006) (Table 1). Antigen presentation is impaired by mutations or downregulation of tumor antigens and major histocompatibility complex (MHC) class I and molecules involved in antigen processing and presentation like transporter associated with antigen processing 1 (TAP1) and low-molecular-mass protein 2 (LMP2) (Igney and Krammer, 2002). The presence of MDSCs and expression of immune suppressive factors and molecules like TGF-β, IL-10, prostaglandins, IDO and tolerance induction by Tregs triggers immune escape. Increased resistance through induction of anti-apoptotic mechanisms also promotes tumor outgrowth. Another mechanism of suppression of NK activity is through downmodulation of NK activating receptors and increased NK inhibitory ligands.

Table 1. Tumor immune escape mechanisms. *Cancer cells have developed a number of mechanisms to circumvent immune destruction. Some of the mechanisms cancer cells use in avoiding immune surveillance are listed. Adapted from (Plate, 2012).*

Pathways Triggered to Escape from Immune Attack	**Microenvironment's Escape Mechanisms**
Decrease MHC expression in tumor cells	ROS (reactive oxygen species), IDO (indoleamine 2,3-dioxygenase)
Induce anergy, decrease antigen presentation	TAM (tumor associated macrophages)
Decrease immune effectors	MDSC (myeloid-derived suppressor cells)
Decreased antigen presenting cell function	Soluble MUC1, VEGF
Induction of T-cell anergy, tolerance and/or suppressed functionally	TGFα, TGFβ, IL-10, VEGF, IDO
Induction of regulatory $CD4^+CD25^+FOXP3^+$ T-cells	Prostaglandin E2, TGFβ, IL-10
Inhibition of T- and NK-cell activity	Soluble MICA/B
Decreased CD3 ζ-chain signaling	ROS, IDO
PD-1 signaled immune cell death	PD-L1/B7-H1, PD-L2/B7-DC
Fas-triggered apoptosis of immune cells	Fas-ligand
Trail-triggered apoptosis	Trail ligand
Decreased T-cell activation, increased memory helper T-cells	B7-H3

2.4. Natural Killer cells (NK)

Natural killer (NK) cells are cytolytic effector cells of innate immune system that has the capacity to lyse the infected or malignant cells 'naturally' without prior antigen priming. They have been recently termed as innate lymphoid cells (ILCs) (Lanier et al., 1986, Spits et al., 2013). The discovery of these large granular lymphocytes dates back to 1975 when they were first identified in mice having lytic activity (Kiessling et al., 1975). Human NK cells comprise approximately 5-15% of all peripheral lymphocytes and are characterized phenotypically by the presence of CD56 and the lack of CD3.

2.4.1. NK cell subsets and function

Based on the cell surface density of CD56 (neural cell adhesion molecule NCAM) and CD16 (low-affinity Fc-receptor γ IIIA), NK cells are divided into two major subsets, CD56 dim constituting the majority of NK cells (90%) and CD56 bright which constitute less than 10% (Figure 6). Functionally these subsets are distinct with the immunoregulatory CD56 bright producing cytokines and CD56 dim which has cytolytic activity and mediate antibody-dependent cellular cytotoxicity (Cooper et al., 2001b, Caligiuri, 2008). NK cells are important players in innate immune response and immune regulators of adaptive immunity. The immunophenotypic profile of different NK cell subsets is represented in Figure 7.

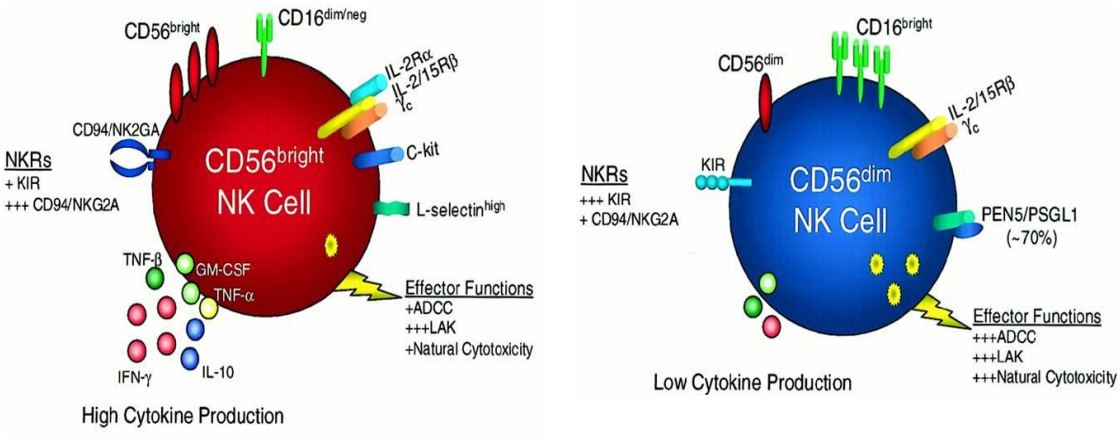


Figure 6. Natural killer cell subsets.*CD56^{bright} and CD56^{dim} NK cell subsets exhibit differential receptor profiles and innate immune functions. Adapted from (Cooper et al., 2001a).*

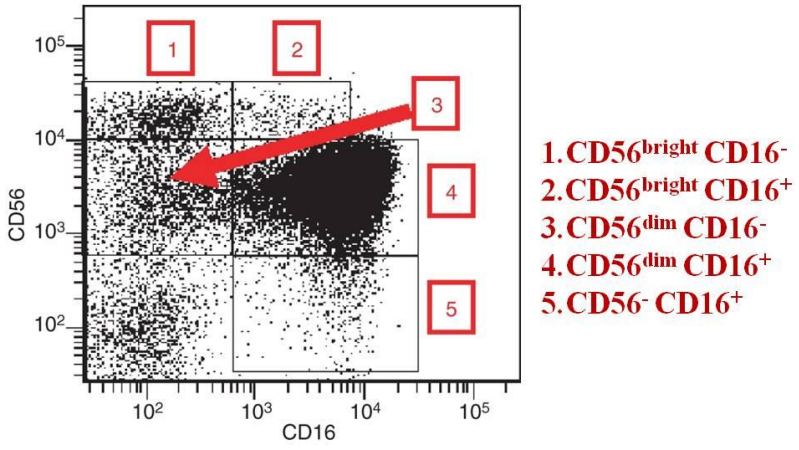


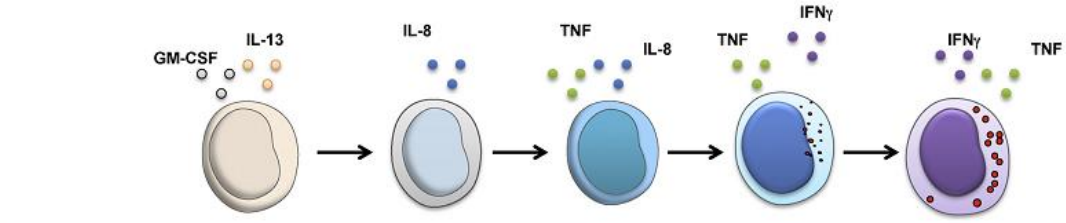
Figure 7. Immunophenotypic profile of NK subsets. *Natural killer cell subsets in human peripheral blood based on the relative expression of CD16 and CD56. A gate was set on CD3^{-} CD19^{-} cells (NK cells) and CD16 expression versus CD56 expression is shown. Adapted from (Poli et al., 2009)*

Regardless of the MHC expression, NK cells can kill virally infected and tumor target cells by releasing cytotoxic granules which are secretory lysosomes containing perforins and granzymes. They form an immunological synapse with the target cells and extrude the content for effective lysis. Degranulation brings CD107a to the surface of the NK cell, and CD107a expression can thus be used as a surrogate marker of NK cell degranulation in flow cytometry assays. They can also kill in a perforin-independent manner using FAS ligand or TRAIL with a slow kinetics. They also produce a number of cytokines and chemokines like IFN-γ, TNF-α, GM-CSF, IL-5, IL-13, CCL3 and CCL5/RANTES.

2.4.2. NK cell development

Bone marrow is the main site of NK development. However they are also present in spleen, lungs, liver and uterus and to a lesser extent in secondary lymphoid tissue (SLT), mucosa-associated lymphoid tissue (MALT) and the thymus. Hematopoietic progenitors differentiate into common lymphoid progenitors that give rise to NK precursors and undergoes different stages of differentiation and maturation to develop into immature NK cells. Maturation into fully functional NK involves the acquisition of activating and inhibitory receptors that regulate NK effector functions. Even though different NK developmental models are evolving studies suggest that suggest that the CD56 dim NK-cell subset is derived directly from the CD56 bright NK (Romagnani et al., 2007). Elucidation of the immunophenotypes of human and mouse NK cell developmental intermediates (NKDIs) that represent distinct maturational stage is

recently evolving. The acquisition of IL-15 also plays an important role in NK cell differentiation and activation. IL-15 selectively promotes NK cell differentiation, functional maturation, and survival (Huntington et al., 2009). Other cytokines like IL-2, IL-4, IL-7, IL-10, IL-12, IL-18, IL-21, IFN and TGFβ influence NK cell maturation (Brady et al., 2010). Maturing NKDIs also acquire functional receptors CD16, CD56, CD94/NKG2A, NKp46, and NKG2D and killer immunoglobulin-like receptors (KIRs) and CD16 (Freud et al., 2006) (Figure 8).



CD34	+	-	-	-	-
CD33	+	+	-	-	-
CD117	++	+	+	-/+	-
CD161	+	+	+	+	+
CD244 (SAP)	+ (-)	+ (-)	+ (-)	+ (+)	+ (+)
CD56	-	+	+	+ (bright)	+ (dim)
NKp44	-	+	+	+	+
NKp46	-	-	+	+	(+)
CD94/NKG2A	-	-	-/+	+	+
LFA-1	-	-	-/+	+	+
CD16	-	-	-	-/(+)	+
KIRs	-	-	-	-	+
CD57	-	-	-	-	-/+
Perforin	-	-	-	-/+	++
Granzymes	-	-	-	-/+	++

Figure 8. Schematic representation of human NK cell differentiation from CD34⁺HSC. *NK cells share a common precursor with other innate lymphoid cells (ILC). Sequential acquisition of markers and functional properties as revealed by in vitro and in vivo studies is represented in the figure. Adapted from (Montaldo et al., 2013).*

Transcription factors involved in NK development

Even though cytokines and surface receptors influence NK development, transcription factors control the fate of NK development. Knockout mice models have helped in studying several TFs involved in the commitment to the NK cell lineage. E4bp4 (E4-binding protein 4), Runx3 (Runt-related transcription factor) and Ets1 (E26 transformation specific TF) acts at early stages of NK development. The E4bp4 expression is detected in NK precursors (NKPs), upregulated in immature NK cells and is maintained in mature NK cells and correlates with IL-15 dependency at different stages of maturation. Ets-1 is required in the early stage of NK cell development to induce the expression of other TFs such as T-bet (T-box transcription factor/TBX21) and Id2 (DNA binding protein inhibitor). In Ets-1-deficient mice, NK development and function are severely impaired. The T-box family T-bet and Eomesodermin (Eomes) are both expressed in mature NK cells (Gordon et al., 2012). CD56 dim NK cells express Blimp1 and it negatively regulates the production of IFN-γ, TNFα, and TNFβ in human NK cells (Kallies et al., 2011). Id proteins are also essential for NK cell development and maturation. Klf4 (Kruppel-like factor 4) is a key TF that determines the progenitor cell fate, and it was reported that loss of Klf4 results in low numbers of NK cells in the blood and spleen (Park et al., 2012). Tox (thymocyte selection-associated HMG box factor) has been shown to regulate NK cell differentiation from hematopoietic stem cells *in vitro* (Yun et al., 2011).

2.4.3. NK cell receptors and ligands

Ly49A was the first inhibitory receptor identified on NK cells in mice and showed that it prevents the elimination of tumor cells expressing specific classical MHC class I molecules (Karlhofer et al., 1992). The activity of NK cells is regulated by receptors with opposing functions, triggering either inhibitory or stimulatory pathways called NK cell receptors (NKRs) (Cooper et al., 2001a, Leibson, 1997). The activating and inhibitory receptors are classified as killer immunoglobulin-like receptors (KIRs) that bind to classical MHC class I ligands (HLA-A, B, and C), the C-type lectin superfamily CD94-NKG2A/C/E heterodimeric receptors that bind the nonclassical MHC class I (HLA-E), natural cytotoxic receptors (NCRs), NKG2D receptors and co-stimulatory receptors. The adequate expression of appropriate KIR ligands protects healthy "self" cells against NK cell reactivity. The major ligands for NKG2D receptors include UL16-binding proteins ULBP1, ULBP2 and ULBP3, and the MHC class-1-chain-related molecules MICA and MICB in humans (Figure 9). The stimulatory pathway is dependent on engagement of activating receptors NKG2D and NCRs like NKp30, NKp44, NKp46, DNAM-1and others when they bind to ligands present on infected or transformed cells, this, in turn, induce NK cell activation and cytotoxicity against the ligand-expressing cells (Lanier, 2001, Moretta et al., 2001).

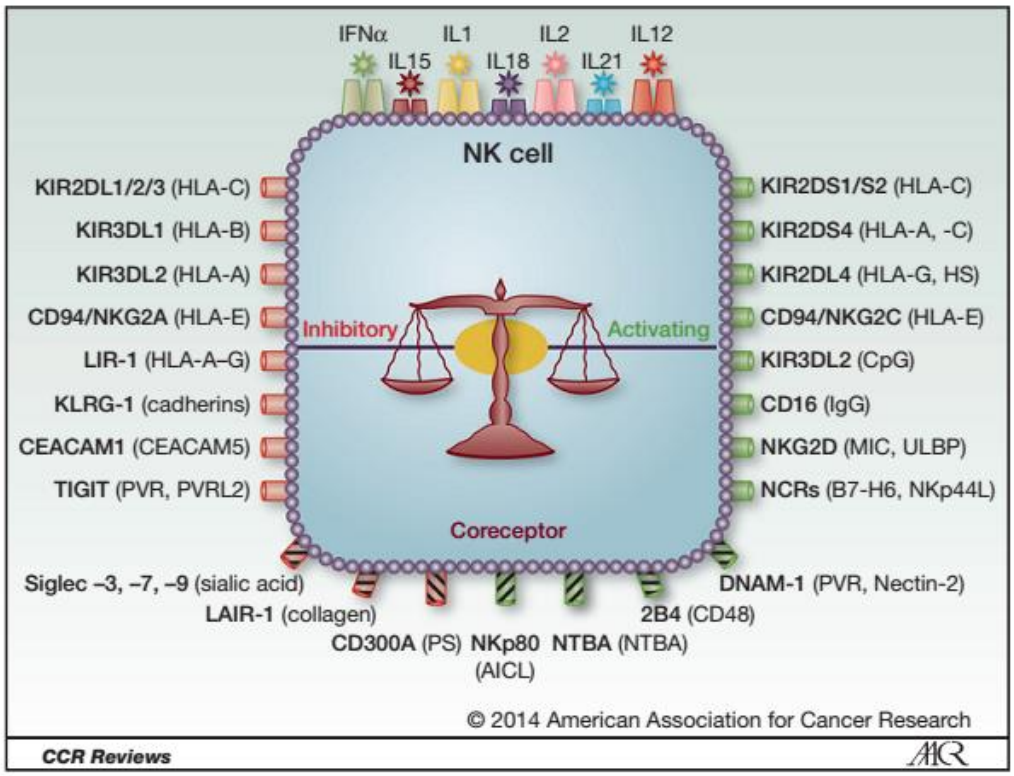


Figure 9. NK cell surface receptors and their ligands. *Cytokine receptors are shown on the top. Other receptors are broadly classified into inhibitory receptors (red), activating receptors (green) and coreceptors (stripes). Their ligands are shown within parentheses. Adapted from (Leung, 2014).*

2.4.4. Missing self-recognition

A unique feature of NK cells is their ability to sense 'missing self' as proposed by Klas Kärre which postulates that NK cells search for self MHC class I and that the failure of finding self-MHC class I results in the failure of inactivation of NK cells and results in target cell lysis (Ljunggren and Karre, 1990) (Figure 10). Engagement of KIRs by their classical MHC class I ligands improves the responsiveness of activating

receptors and ‘arms’ these cells to become competent effector cells which is termed as NK cell education or NK licensing or NK arming (Kim et al., 2005).

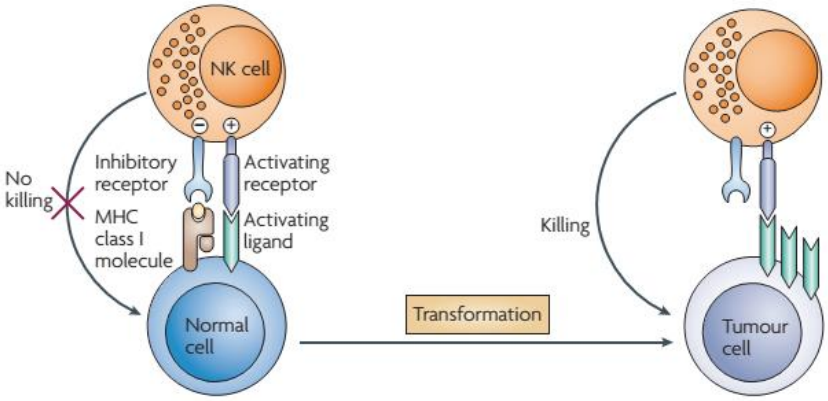


Figure 10. Missing self-recognition of NK cells. *A system used by natural killer cells to recognize normal cells and cells that lack MHC class I surface molecules. NK cell activation is regulated by a balance between signals mediated through activating and inhibitory receptors. Upon cellular transformation, MHC class I ligands for inhibitory receptors are often reduced or lost resulting in NK activation and target cell killing. During tumor progression, tumor variants may evolve that upregulate ligands for inhibitory receptors and lose ligands for activating receptors. These tumors may escape NK-cell-mediated recognition. Adapted from (Ljunggren and Karre, 1990)*

2.4.5. KIR haplotypes

Human KIR haplotypes are diversified by gene content and allelic polymorphism. The genes that encode the inhibitory HLA-C receptors (KIR2DL1, KIR2DL2 and KIR2DL3) are situated in the centromeric part of the locus, whereas the genes that encode the inhibitory HLA-B receptor (KIR3DL1) and HLA-A receptor

(KIR3DL2) are in the telomeric part. KIR haplotypes comprise two groups, A and B, according to the genes they contain (Parham, 2005). Haplotype A is defined by the presence of 2DL1, 2DL3, 3DL1, 2DS4 and 2DP1 along with framework genes and haplotype B by the presence of one or more genes encoding activating KIRs, KIR2DS1/2/3/5, KIR3DS1 and inhibitory KIRs, KIR2DL5A/B and KIR2DL2 (Middleton and Gonzelez, 2009) (Figure 11). Distinctive patterns of KIR expression could assist diagnosis of certain tumors. It has been shown that leukemic patients display a more inhibitory AB haplotype and also has increased frequency of KIR2DL2 and KIR2DS2 compared to healthy controls (Verheyden et al., 2004).

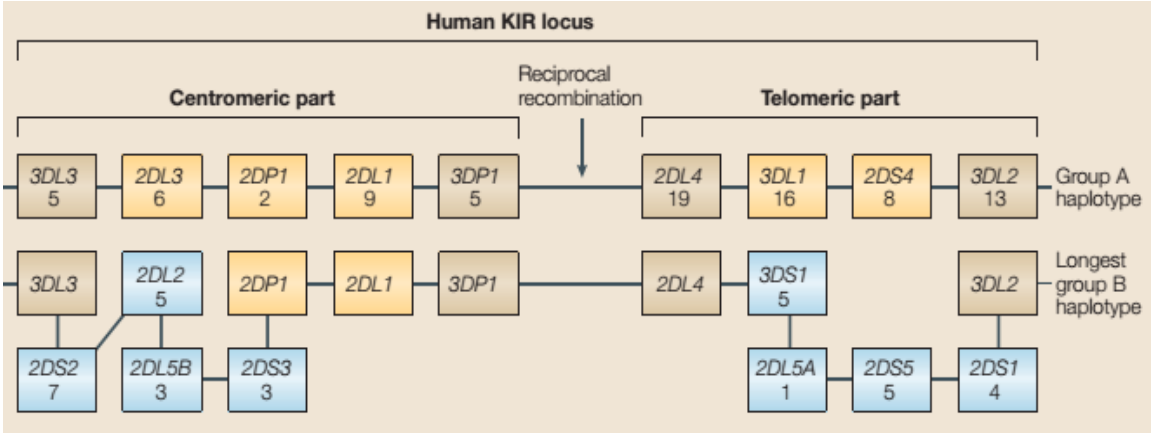


Figure 11. Organization of the human KIR locus. *KIR locus is on the human chromosome region 19q13.4. Conserved genes are shown in brown. Genes that can be present in both group A and group B KIR haplotypes are shown in yellow, and genes and/or alleles that are specific to group B KIR haplotypes are shown in blue. KIR3DL3 and KIR3DP1 delimit the centromeric part of the KIR locus, whereas KIR2DL4 and KIR3DL2 delimit the telomeric region. KIR2DP1 and KIR3DP1 form the two pseudogenes. Adapted from (Parham, 2005).*

2.4.6. Clinical applications of NK cells

The progress in our understanding of NK cell immunobiology has paved the way for novel NK cell-based therapeutic strategies for the treatment of cancer. NK cells are already linked to a number of clinical trials for successful outcomes after hematopoietic stem cell transplantation (HSCT). The capacity of NK cells to kill leukemic cells has been demonstrated in patients with AML receiving haplotype-mismatched stem cell transplants (Giebel et al., 2003, Ruggeri et al., 2002). Activated NK cells have been known to be effective in killing a broad range of tumor targets. Clinical data on the efficacy of NK cell therapy in leukemia is well known in hematological malignancies (Miller et al., 2005).

Various studies have looked at immunotherapeutic strategies such as upregulation of functional activating receptors on NK cells to improve NK cell cytotoxicity (Berneman et al., 2009). IL-15 is one such cytokine which can up-regulate the expression of the NK activating receptors and increase the NK lytic activity in patients with AML (Boyiadzis et al., 2008). Another scenario for promoting NK cell cytotoxicity may be achieved by modulation of the tumor cells, rendering them more susceptible to NK cell recognition. An attractive immunotherapy would be to up-regulate these ligands on leukemic cells to enhance their susceptibility to NK cell recognition and restore their sensitivity to NK cell killing. Adoptive transfer of *ex vivo* activated or expanded allogeneic NK cells has emerged as a promising immunotherapeutic strategy for cancer. NK cell lines, such as NK-92 and KHYG-1,

have been documented to exert antitumor activity in both preclinical and clinical settings (Tonn et al., 2013). The feasibility of genetically engineered NK cells to express CARs (chimeric antigen receptor) and other engager-modified NK cells has also been shown in the preclinical setting, but there are safety concerns regarding the adoptive transfer using these cells (Tettamanti et al., 2013).

2.5. Myeloid-derived suppressor cells (MDSC)

One of the possible mechanisms of failure of the immune system to inhibit tumor growth is the accumulation of $CD11b^{+}Gr1^{+}$ immature myeloid cells. This heterogeneous population termed as MDSCs in 2007 has become the focus of study in recent years and is found to accumulate in bone marrow, spleen, blood and tumor sites in patients and experimental animals with neoplasia and thought to have a robust immunosuppressive activity. Morphological, phenotypic and functional heterogeneity is a hallmark of MDSC, but there exists an ambiguity in the defining the phenotype of MDSC. This population is first identified in tumor-bearing mice with demonstrated abilities to suppress CD8+T-cell antitumor immunity. MDSC accumulate in large numbers during many pathologic conditions, including cancer, infectious diseases, trauma, sepsis, etc (Gabrilovich and Nagaraj, 2009).

Studies implicated up-regulation of arginase, nitric oxide (NO), and reactive oxygen species (ROS) as the major factors responsible for the immune suppressive activity of MDSC. Initially, MDSC in mice were defined as cells expressing a

$Gr1^{+}CD11b^{+}$ phenotype and lacking the expression of markers typical of mature macrophages and DCs. In humans, MDSC were defined as immature myeloid cells, lacked markers of lymphocytes, natural killer cells, monocytes, and DC, and express myeloid cell markers CD33 and CD11b, as well as, in some studies, granulocyte markers. Currently, MDSC in mice and men can be subdivided into two major groups: granulocytic MDSC (G-MDSC) and monocytic MDSC (M-MDSC). In mice, G-MDSC has a phenotype of $CD11b^{+}Ly6G^{+}Ly6C^{low}$, whereas M-MDSC has a phenotype of $CD11b^{+}Ly6G^{-}Ly6C^{high}$.Two significant subsets of human MDSCs have been reported: granulocytic or polymorphonuclear MDSCs (Gr-MDSCs or PMN-MDSCs) with $CD11b^{+}CD33^{+}CD14^{-}HLADR^{-}$ phenotype and monocytic MDSCs (Mo-MDSCs or M-MDSCs) that are $CD14^{+}HLADR^{-}$ (Gabrilovich and Nagaraj, 2009).

G-MDSC primarily uses ROS as the mechanism of immune suppression. M-MDSC primarily use up-regulation of inducible nitric oxide synthase (iNOS), arginase and an array of immune suppressive cytokines to suppress various immune functions (Gabrilovich et al., 2012). MDSCs are immature myeloid cells that have acquired potent immune suppressive activity and other non-immunological functions (Figure 12). They also have impaired the ability to differentiate into mature myeloid cells, and as a result, accumulate in peripheral lymphoid organs. It is also shown that these cells do not exist in healthy individuals or control mice.

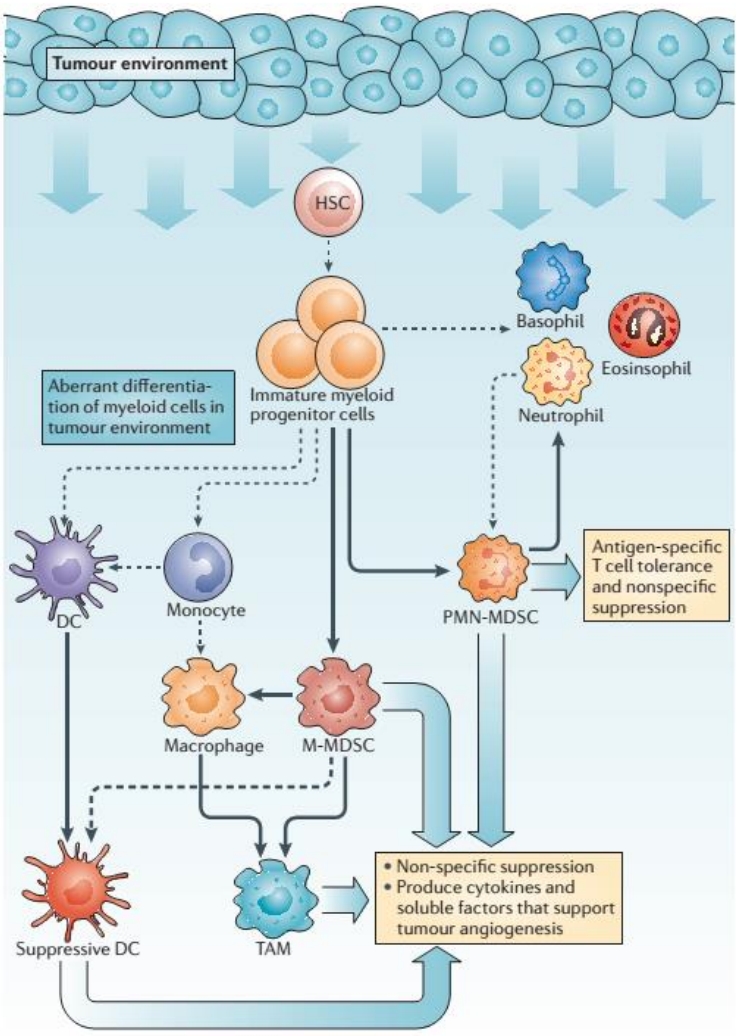


Figure 12. Immature myeloid cells or MDSCs. *Changes that occur in myeloid cells in cancer is depicted in this figure. Factors produced in the tumor microenvironment by tumor cells and stromal cells promote the aberrant differentiation of myeloid lineage cells. The dotted lines show the normal pathways of myeloid cell differentiation from immature myeloid precursor cells to dendritic cells (DCs), macrophages and granulocytes. The solid bold lines indicate the aberrant pathways of myeloid cell differentiation that occur in cancer, in which the tumor environment can promote the development of various immunosuppressive populations like M-MDSCs, PMN-MDSCs and TAMs. Adapted from (Gabrilovich et al., 2012).*

MDSCs in cancer

MDSCs are a relatively novel population of myeloid-derived immunomodulatory cells which have been reported to be associated with poor prognosis in solid tumors. Some studies have shown increased MDSC proportion in patients who develop acute graft-versus-host disease (aGVHD) after allogeneic hematopoietic stem cell transplantation (Vendramin et al., 2014). There is very limited data on the role of MDSCs in leukemia. A recent study showed an increase of the Gr-MDSCs subset in chronic myeloid leukemia (CML) patients, and they found that these subsets are significantly higher at diagnosis compared to healthy controls and decreased to normal levels after imatinib therapy (Giallongo et al., 2014).There is no reported data evaluating the proportion of these cells in newly diagnosed acute leukemia and their association with conventional risk factors in acute leukemia.

2.6. Immunological aspects of Leukemia

2.6.1.Mechanisms of immune escape in leukemia

Much remains unknown regarding the immune escape mechanisms in leukemia and the host immune response may be different from those in solid tumors. Leukemic cells are made susceptible to T cell and NK cell attack by the expression of leukemia-associated antigens, MHC and co-stimulatory molecules and ligands for activating receptors. Leukemic cells can circumvent this immune surveillance resulting in a

dysfunctional anti-leukemic immune response. Leukemic cells can escape even the most powerful antigen-driven T-cell suppression.

There are accumulating evidence of NK contributing to anti-tumor immune response. It is hypothesized that leukemic cells may have evolved various escape mechanisms from immune surveillance by the quantitative and qualitative defects in NK receptors and ligands. Human epithelial tumors shed the Major Histocompatibility Complex class 1 related chains A and B (MIC A/B) ligands, which accumulate in serum, causing the down-regulation of activating receptors such as NKG2D (Groh et al., 2002). Several lines of evidence indicate that the expression level of NKG2D ligands (NKG2DL) like MIC A/B and UL16 binding proteins (ULBPs) on leukemic cells affecting its sensitivity to killing by NK cells. Low levels of triggering ligands may impair the clearance of leukemia by NK and T cells bearing the activating NKG2D and NCR receptors (Nowbakht et al., 2005).

In AML, the downregulation of NCRs NKp30 and NKp46 is hypothesized as the underlying cause of the low susceptibility of leukemic blasts to lysis by autologous NK cells (Costello et al., 2002). NCR dull NK cell phenotype was associated with poor survival in AML patients (Fauriat et al., 2007). In 1984, Kerndrup et al. noticed that the decreased NK activity seen in patients with MDS was caused by a decreased number of circulating NK cells (Kerndrup et al., 1984). Recently there is developing evidence on the role of KIR gene polymorphism and predisposition to leukemia. Studies have shown strong inhibition mediated by KIR3DL1-Bw4 interactions in chronic lymphocytic

leukemia (CLL) and KIR2DL2-Cw1 in myeloid leukemia stating the dominance of inhibitory over activating KIR signals. (Verheyden and Demanet, 2006).

The best evidence for functional disturbance of T cells in AML is the altered expression of the TCR signaling complex. Buggins et al. reported abnormal expression of CD3-zeta in 64 % of patients with myeloid malignancies and successful remission induction was associated with recovery of CD3-zeta expression (Buggins et al., 1998). Another potent T-cell suppressive MDSCs are found to be increased in PB of patients with various malignancies (Giallongo et al., 2014, Sun et al., 2015). Pre-B ALL cells are known for poor expression of co-stimulatory molecules thus elicits a weak immune host response. A study shows the mechanisms of resistance of B-ALL cells to NK-mediated cytotoxicity by the absence of MICA, MICB or ULBPs (or only at very low levels) in resistant B-ALL cell lines (Romanski et al., 2005). Tregs also alter the function of DCs and make them remain immature and more tolerogenic. Immature DCs express indoleamine 2,3-dioxygenase (IDO), causing immunosuppression and promoting T-cell apoptosis. AML patients with high IDO mRNA expression levels in the leukemic blasts were correlated with poor disease-free survival and OS (Chamuleau et al., 2008).

Recent studies highlight the importance of immune check points like the expression of PD-L1 and CTLA-4 ligands on cancer cells inhibiting cytotoxic lymphocytes. In AML, PD-L1 expression by AML blasts has been shown to protect AML cells from killing by cytotoxic T cells (Kronig et al., 2014). CD200 is a protein belonging to the immunoglobulin superfamily, which has been associated with a poor

prognosis in lymphoproliferative disorders and in acute leukemia which causes direct suppression of memory T-cell function (Tonks et al., 2007). Several soluble factors produced by hematologic malignant cells or stromal cells within the tumor environment significantly contribute to impair the survival and functions of effector cells and preserve the immunosuppressive environment. Cytokines such as IL-10, TGF-β, and VEGF are associated with disease progression and poor survival in several hematologic malignancies (Ramsay and Gribben, 2009). Several reports indicate that leukemic cells harboring class II-associated invariant chain peptide (CLIP) can serve as an immune escape mechanism in AML by disturbing the activation of tumor-reactive CD4+ T cells (Chamuleau et al., 2004). The metabolic activity and alteration in the metabolic program of immune cells, and in particular in T cells and NKs, is gaining significant attention. Glucose deprivation causes severe impairment of the proliferation and effector functions of T cells (Jacobs et al., 2008). Hypoxia impairs T and NK cell proliferation, its cytolytic activity and the expression of activating receptors and cytokine secretion, which exacerbates the immunosuppression (Balsamo et al., 2013). Some of the immune suppressive cells and probable mechanisms immune dysfunctions are depicted in Figure 13.

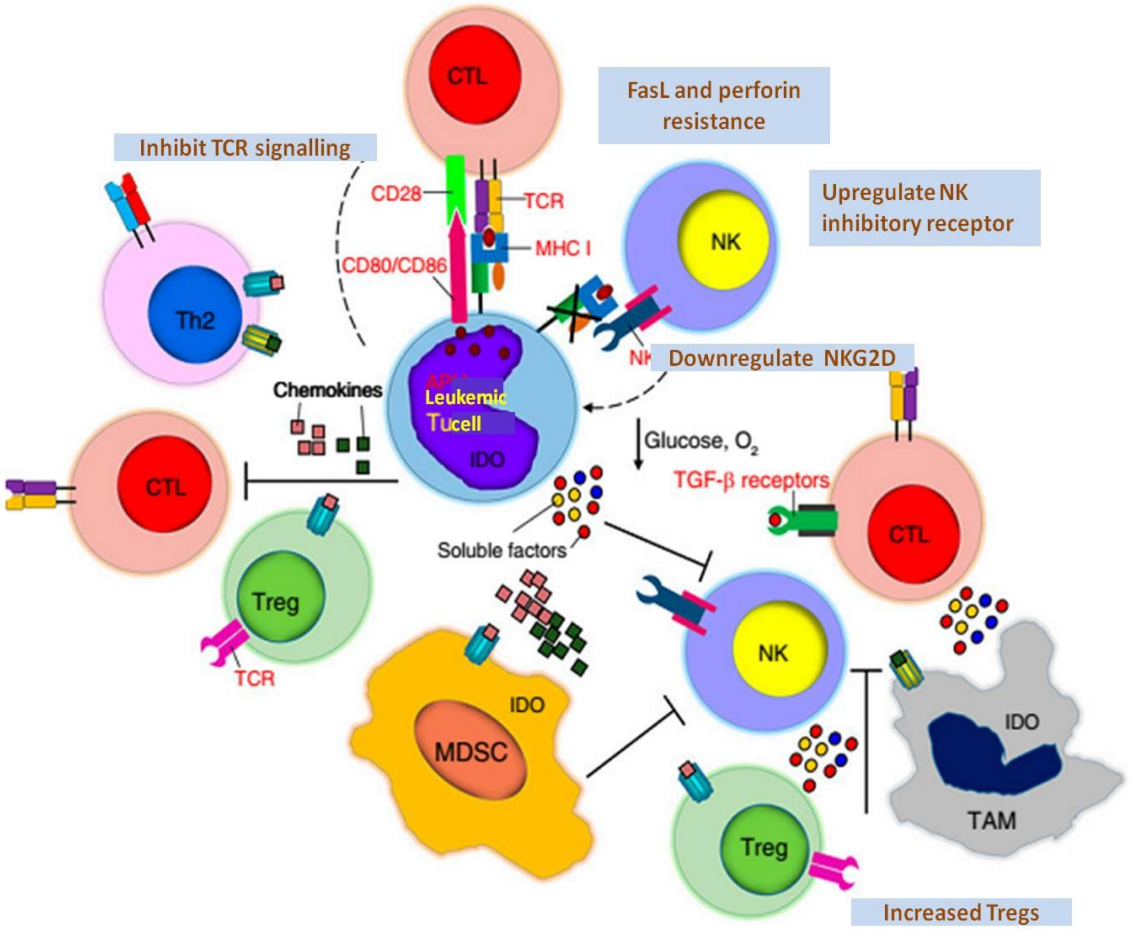


Figure 13. Leukemic cell promoting immune dysfunctions. *Leukemic cells are surrounded by several cell subsets including Tregs, TAMs, and MDSCs, which hamper the function of cytotoxic T cells and NK cells. Chemokines play a critical role in recruiting inhibitory cells, whereas soluble factors such as TGFβ, nutrient deprivation, and hypoxia impair the proliferation and function of effector cells. Modified from (Sun et al., 2016)*

2.6.2. Chemotherapeutic agents modulating immune response

Although cancer chemotherapy has historically been considered immune suppressive, it is known that certain chemotherapies can augment tumor immunity. Approaches to stimulate anti-leukemia immunity is of interest in recent years. Conventional anticancer chemotherapy is thought to act through the killing of tumor cells or by irreversibly arresting their growth with the fact that cytotoxic drugs interfere with DNA synthesis and replication. Accumulating evidence indicates that several chemotherapeutic drugs are more efficient against tumors that are implanted in immunocompetence environment for a better clinical outcome. Anti-cancer chemotherapy can be simultaneously lymphodepleting and immunostimulatory. Cytotoxic drugs such as cisplatin, doxorubicin, mitomycin C and camptothecin also primed tumor cells to elimination by immune cells, including natural killer or cytotoxic T lymphocytes (Lacour et al., 2001). A DNA alkylating agent such as cyclophosphamide might act via the depletion of regulatory T cells. Gemcitabine, an antimetabolite agent, attenuates the immunosuppressive properties of the tumor microenvironment by eliminating MDSCs. Some chemotherapeutics, including anthracyclines, oxaliplatin and cyclophosphamide (CTX) are unique in their capacity to induce an immunogenic type of tumor cell death by releasing immunogenic cell determinants (ICD) such as calreticulins, HMGB1(high mobility group box1 protein) and the release of ATP, thus enhancing the uptake of tumor antigens by dendritic cells (Kepp et al., 2009).

Various agents have been evaluated for their ability to induce ligands on leukemic cells to augment the immune-mediated anti-leukemic effect. All-trans-retinoic acid and the histone deacetylase inhibitor sodium valproate were repeatedly shown to induce NKG2D ligand expression in certain types of tumors, including AML (Diermayr et al., 2008, Poggi et al., 2009). The proteasome inhibitor bortezomib and histone deacetylase inhibitors were also shown to improve NK-cell-mediated killing of AML cells through the TRAIL pathway (Hallett et al., 2008). ATO has shown to increase the surface expression and transcription of NKG2D ligands predominantly ULBP1 in K562, NB4 and MCF7 cell lines and has shown increased susceptibility of cancer cells to NK-mediated cytotoxicity on exposure to ATO which suggests the immunomodulatory property of ATO to improve the effectiveness of NK cell-based cancer immunotherapy (Kim et al., 2008). The role of KIR function and genetic polymorphism for recognition of leukemia and its impact will be useful for developing novel concepts for NK cell-based immunotherapy strategies (Venstrom et al., 2012). Drugs which are known to effectively combined with adjuvant NK cell– based immunotherapy are asparaginase, bevacizumab, bleomycin, doxorubicin, epirubicin, etoposide, 5-fluorouracil, hydroxyurea, streptozocin, and 6-mercaptopurine (Markasz et al., 2007) (Figure 14). Lenalidomide and Pomalidomide are thalidomide derivatives known as immunomodulatory derivatives (IMIDs) known to have the ability to modulate immune responses including stimulation of NK cell and T cell activity in multiple myeloma cells (Davies et al., 2001).

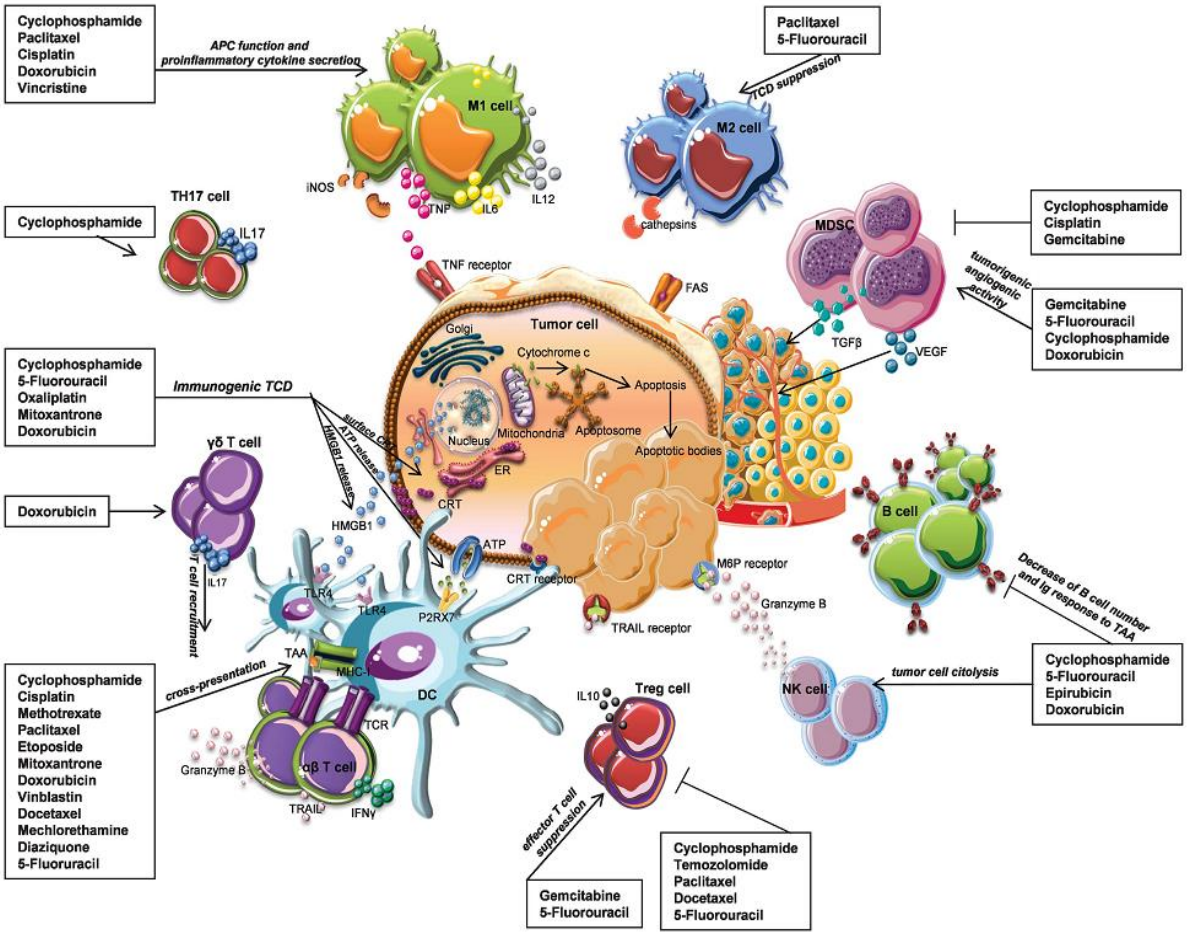


Figure 14. Immunomodulation by cytotoxic drugs. *Conventional antineoplastic drugs can activate anticancer immune responses through different mechanisms: (i) the inhibition of tumor-induced suppressive mechanisms, (ii) the direct stimulation of T and B cell responses, (iii) the enhancement of tumor immune visibility by cytotoxic cell subsets or phagocytes. Low-dose CTX and gemcitabine deplete Tregs or MDSCs. Paclitaxel, cisplatin and doxorubicin induce the upregulation of mannose-6-phosphate receptors on the surface of tumor cells, rendering them permeable to granzyme B. Paclitaxel induces proinflammatory cytokines secretion from macrophages, leading to DC, NK and T-cell activation. Anthracyclines, oxaliplatin and CTX promote tumor expression of calreticulin, and release of HMGB1 and ATP by dying tumor cells, thus stimulating phagocytosis and cross-presentation of antigens by DC. Adapted from (Bracci et al., 2014)*

When the bulk disease is reduced by chemotherapy, the goal of immunotherapy is to stimulate the immune system and provide durable remission. The first successful immunotherapy was documented in 1890 when Coley demonstrated bacterial products (Coley toxins) to treat bladder cancer. Immunotherapy including vaccines and immune checkpoint blockade is the newest class of systemic cancer therapies. Several different types of immunotherapy are currently being explored for the treatment of leukemia including adoptive cell therapy, monoclonal antibodies, checkpoint inhibitors, therapeutic vaccines, adjuvant immunotherapies and cytokines. Appropriate therapeutic regimens must be designed, both the dosage and the order of administration of chemotherapeutic agents relative to immunotherapeutic interventions which have been shown to influence the success of immunochemotherapy.

2.6.3. Immune reconstitution post chemotherapy

Even though recent advances in chemotherapy has benefited patients regarding longevity, one of the major side effects of chemotherapy is immunosuppression, and hence it is important that immune reconstitution should be accomplished rapidly following cessation of chemotherapy. Immune reconstitution is an area of intense research in HSCT and has been studied extensively, and persistent immune defects may be directly related to severe post-transplant infections, relapse or secondary malignancies. However, there is little knowledge concerning long-term recovery of the immune subsets post chemotherapy in leukemia, and it will differ quantitatively and qualitatively from HSCT. Behl et al. evaluated the impact of lymphocyte recovery after

induction chemotherapy on OS and leukemia-free survival (LFS) in newly diagnosed AML patients. Absolute lymphocyte recovery (ALC) was studied at days 15, 21, 28 after induction chemotherapy and before the first consolidation chemotherapy. Superior leukemia-free survival (LFS) were observed at all time points when ALC greater than or equal to 500 cells/μl (Behl et al., 2006). Other investigators also report an association of good ALC recovery with more favorable outcome in children with acute lymphoblastic leukemia (ALL) and AML after chemotherapy (De Angulo et al., 2008). Cytotoxic antineoplastic therapy is primarily related to T-cell depletion, with CD4 depletion more severe than CD8 depletion (Mackall et al., 1997). A study in AML patients in CR showing a low number of $CD4^+$ and $CD45RA^+T$ cells at an early period after chemotherapy and they have recovered to normal levels in 2 years (Ohnishi et al., 1998). In children with AML and ALL, the memory subpopulations of T and B cell compartments recovered more slowly and remained decreased even post chemotherapy (van Tilburg et al., 2011).

Alanko et al. showed that the reconstitution of B cells and immunoglobulin was accomplished within six months after discontinuation of therapy in childhood ALL, indicating sufficient function of the immune system (Alanko et al., 1992). Another study documented persistent immunosuppression in children with ALL during maintenance therapy. Reconstitution of B lymphocytes and natural killer cells occurs early while T cell reconstitution shows delayed recovery of both T helper and T

suppressor cells but immunosuppression during maintenance therapy has no major clinical impact in terms of increased incidence (El-Chennawi et al., 2008).

Accumulating evidence indicates that $CD4^+CD25^+$ T regulatory cells are essential for the maintenance of self-tolerance and immunosuppression. Elevated frequencies of $CD4^+CD25^+$T cells are found in peripheral blood of AML patients at diagnosis when compared with controls and are associated with poor prognosis (Szczepanski et al., 2009, Shenghui et al., 2011). NK cells were known to reconstitute rapidly post transplant (Rajasekar et al., 2009), but studies in pediatric ALL showed decreased NK cell counts compared to controls during the therapy and recovered rapidly post chemotherapy. Despite normal counts, NK cell function may be impaired for several months (Alanko et al., 1995). Another study in AML patients showed a greater frequency of immature NK cells during first complete remission (Dauguet et al., 2011). NKT cells are a distinct subset of T cells that share the NK characteristics and rapidly produce an array of cytokines upon activation and are crucial for antitumor immunity. The low number of NKT cells was shown to be associated with poor prognosis in AML (Najera Chuc et al., 2012).

DCs are potent antigen-presenting cells that are essential for the initiation of primary cellular immune responses. They derive their potency from the rich expression of MHC class I, class II, costimulatory and adhesion molecules necessary for the activation of antigen-specific T cell responses. The nature of the recovery of dendritic cells (DC) has not been explored well in HSCT and in hematological malignancies. A

quantitative imbalance in circulating blood monocytiod DCs (MCDC) and plasmacytoid DCs (PCDC) has been observed in AML patients (Mohty et al., 2001). Defective DCs are found in the peripheral blood of patients with AML and can induce tolerance towards leukemic cells (Mohty et al., 2001). Studies from Maecker et al. showed decreased numbers of MCDC and PCDC in PB of pediatric B-ALL (Maecker et al., 2006).

2.7. Biology of Acute promyelocytic leukemia

Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia with distinct molecular, clinical features and laboratory features at presentation. It is characterized by the presence of a balanced reciprocal translocation involving a portion of the retinoic acid receptor alpha gene (RARα) on chromosome 17 and a portion of a partner gene which in 95% of cases is the PML gene on chromosome 15, denoted as t(15;17)(q24.1;q21.2) resulting in PML/RARα gene fusion and chimeric protein (Sirulnik et al., 2003). PML-RARα is a transcriptional repressor with gain-of-function and dominant-negative properties, resulting in transcriptional repression of retinoic acid and non-retinoic acid target genes thus resulting in inhibition of myeloid differentiation (Kamashev et al., 2004, van Wageningen et al., 2008). APL is biologically, clinically and prognostically different from other subtypes of AML. Historically APL has been associated with distinct coagulopathy resulting in DIC with a high incidence of early hemorrhagic death (Stein et al., 2009).

The first report on APL traces back to 1957, when a Norwegian hematologist L.K Hillestad reported three cases of a special type of acute myelogenous leukemia whose 'outstanding feature was its very rapidly progressing disease of few weeks duration, a white blood cell picture dominated by promyelocytes and severe bleeding caused mainly by fibrinolysis', thus recognized APL as a distinct clinical entity (Hillestad, 1957). J Bernard (Hôpital St. Louis, Paris) in 1959 gave a detailed account of APL in a series of 20 patients with clinical manifestations of the disease and its association with promyelocytic proliferation, hyperacute onset and catastrophic hemorrhagic events.

Acute promyelocytic leukemia (APL) accounts for 5 to 15 percent of all adult leukemias (Ribeiro and Rego, 2006). About 30,800 cases of acute leukemia are diagnosed yearly in the United States; about 1000 of these were acute promyelocytic leukemia (APL). The median age of onset is about 40 years and the incidence is very low in children under 10 years of age. Some reports indicate a higher incidence in Hispanics and a lower incidence in blacks and the incidence of APL does not vary by gender. Asians did not differ from non-Hispanic whites in lifetime or age-specific incidence rates (Matasar et al., 2006). Secondary APL or therapy related APL can also emerge after chemotherapy or radiotherapy for neoplastic or non-neoplastic disorders (eg, breast and prostate carcinoma, lymphoma, other solid tumors), especially in association with the use of topoisomerase-II inhibitors such as etoposide and doxorubicin, mitoxantrone for multiple sclerosis (Beaumont et al., 2003).

2.7.1. APL Pathogenesis

The first reports of the translocation in APL came in 1967 were Engel et al. observed a deletion of long arm of chromosome 17 or 18 and further, the translocation of chromosomes 15 and 17 was observed as a consistent chromosomal change in APL by Rowley et al. in 1977 (Rowley et al., 1977). In 1991, it was discovered that RARα fuses with a novel transcription factor PML (de The et al., 1990, Goddard et al., 1991). PML-RARα fusion protein plays a central role in the initiation of leukemogenesis by exerting a dual dominant negative function on PML and RARα pathways.

RARα is a RA-responsive transcription factor which binds to specific retinoic acid response elements (RARE) at the promoter region of target genes involved in myeloid differentiation. RARα binds to RARE through the highly conserved C domain containing two C2C2 zinc finger motifs. When the RA ligand is not present, RARα forms heterodimers with the retinoid X receptor (RXR) and the AF2 domain of RARα associates with the co-repressor complex (NCOR/ SMRT/Sin3A) along with histone deacetylase (HDAC) and induces transcriptional repression. Thus normal RARα transcriptional activity is inhibited. Physiological concentrations of RA (1×10^{-9}M) can release the nuclear co-repressors complex from the RAR-RXR and recruit coactivators with histone acetyltransferase activities (HAT). This results in hyperacetylation of histones at RARE sites, open up chromatin and activate transcription of RARα-target genes (Dilworth and Chambon, 2001, Glass and Rosenfeld, 2000). However, PML-RARα shows a higher avidity for repressors having two strong binding sites, so a higher

concentration of RA is required to release the corepressors (pharmacological doses of 1 $\times 10^{-6}$M) when compared to wild-type RARα (Figure 15).

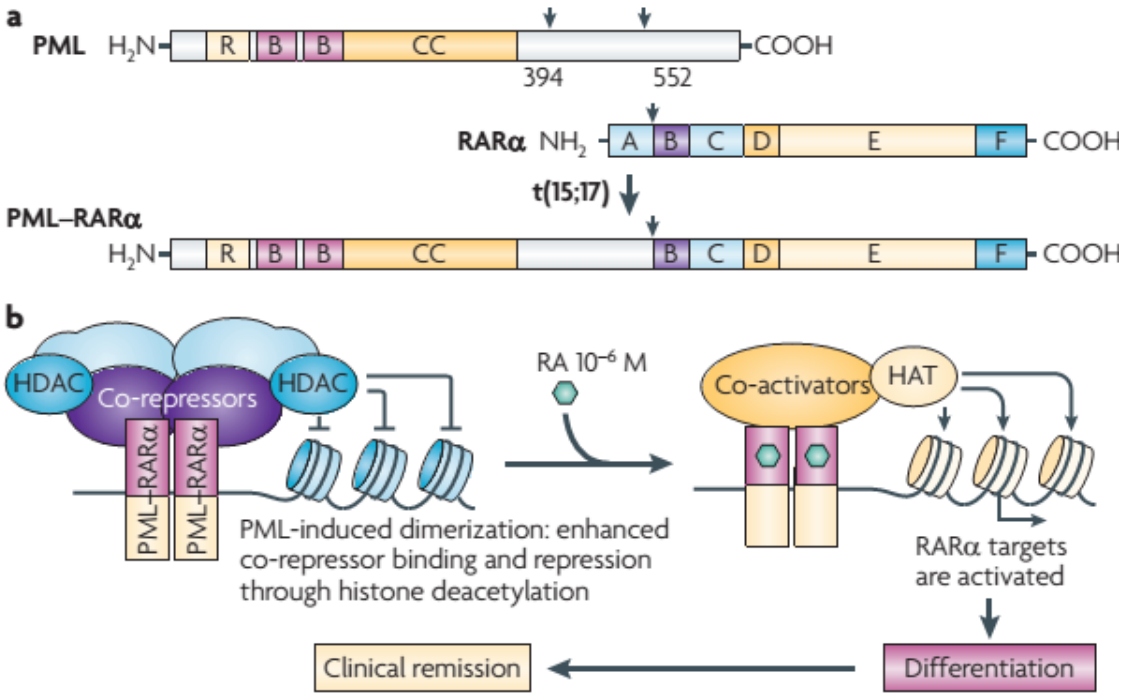


Figure 15.The classical model of APL pathogenesis. *a) The structure of PML and RARα proteins, together with the PML-RARα fusion. RING (R), B boxes (B) and coiled-coil (CC) domains in PML are indicated. The RARα DNA-binding domain (C) and hormone-binding domain (E) are shown. A, B, D and F are other regulatory domains. PML–RARα retains the functional domains of both proteins, allowing dominant-negative activities on both PML and RARα. b) PML–RARα homodimers bind and repress RARα targets through enhanced recruitment of co-repressors. Retinoic acid (RA) converts PML–RARα into an activator and restores differentiation, yielding clinical remissions. Adapted from (de The and Chen, 2010).*

PML is a nuclear matrix protein with a cysteine-rich region (aa 57-222) composed of zinc-finger structures like RING finger, B-box motifs and coiled-coil motifs (RBCC for RING-B Box-Coiled-Coil) helps PML to localize to discrete nuclear domains called PODs (PML oncogenic domains) or NB (nuclear bodies) with 0.1-1μM in size and around 1 to 30 bodies per cell. In t(15;17) APL cells, PML is delocalized from the NBs to a micro speckled nuclear pattern and relocalizes to the NB after ATRA treatment (Weis et al., 1994). Even though the exact function of PML in not clearly understood, it appears to have pro-apoptotic activity and mutations in the RING finger or B box cysteine residues result in loss of nuclear body localization and growth suppressor function.

Based on the location of breakpoints within the PML site, there are three subtypes for PML/RARα transcript:bcr1, bcr2, and bcr3. Of these, bcr 1 derived from breakpoints in PML intron 6 which constitute 55-60% cases, bcr3 transcript derived from breakpoints in PML intron 3 constituting 35-40% and brc2 results from breakpoint in PML exon 6 in approximately 8% of cases which all get fused to RARα intron 2 (van Dongen et al., 1999) (Figure 16). The bcr1 and bcr2 are together referred to as long (L) isoform and bcr3 as short (S) isoform. In > 95% of morphologically defined APLs, the PML/RARα fusion is detectable by fluorescence in situ hybridization (FISH) or reverse-transcriptase polymerase chain reaction (RT-PCR), while in the remaining cases several variant rearrangements have been described that constantly involve RARα and partner genes other than PML.(Mistry et al., 2003). Those fusion genes may involve at low

frequency (< 3%) like the promyelocytic leukemia zinc finger (PLZF), or very rarely the nucleophosmin (NPM), nuclear mitotic apparatus (NUMA1), and signal transducer and activator of transcription 5b (*STAT5B*), cAMP-dependent protein kinase type I-α regulatory subunit (*PRKAR1A*), FIP1-like 1 (*FIP1L1*), or BCL6 corepressor (*BCOR*) partner genes (Redner, 2002, Kondo et al., 2008). The nature of the fusion partner is important to disease biology particularly with respect to all-trans retinoic acid (ATRA) sensitivity, were the involvement of PLZF and STAT5b being characterized by retinoid resistance (Arnould et al., 1999).

PML-RARα is not sufficient enough to cause APL. In the murine APL models, the incomplete penetrance, long latency, and additional cytogenetic changes accompanying the onset and progression of the disease strongly suggest that additional mutations are required for the development of APL, like FLT3, KRAS or WT1 (Greif et al., 2011).

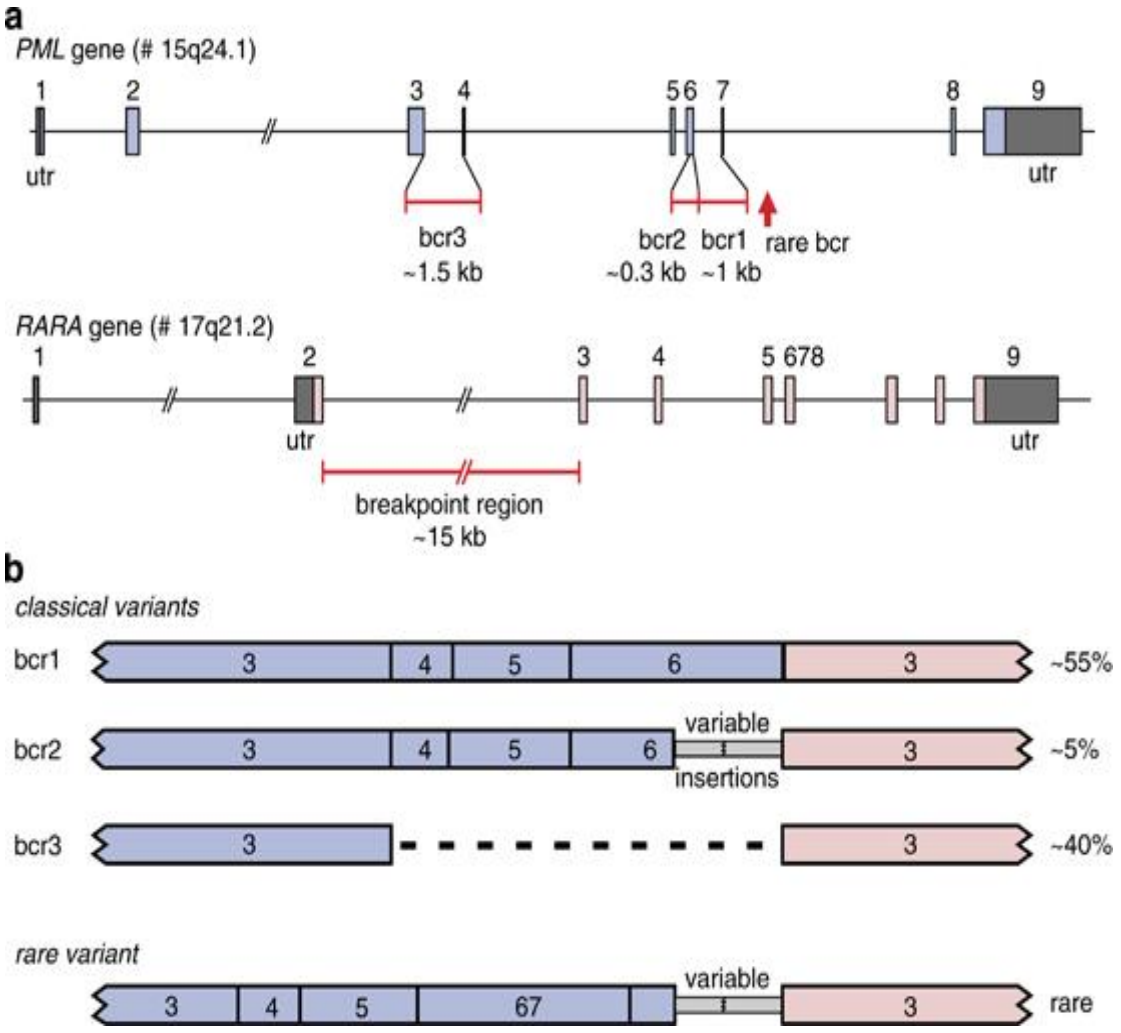


Figure 16. The structure of the PML and RARα genes with the break point regions and the corresponding fusion gene transcripts. *(a) The break point cluster regions (bcr's) in PML are bcr1 in intron 6, bcr2 in the downstream part of exon 6 and bcr3 in intron 3. In the RARα gene the break points cluster is in intron 2. (b) The three well-defined bcr's and the rare intron 7 breaks in the PML gene result in four different PML–RARα fusion transcripts. The relative frequency of the various PML–RARα variants is given in percentages. Adapted from (van Dongen et al., 1999, Dekking et al., 2012)*

2.7.2. Clinical presentation and diagnosis

APL was classified as AML-M3 in the older French-American-British (FAB) classification system and is currently classified in WHO classification as acute promyelocytic leukemia with t(15;17)(q24.1;q21.2). APL represents a medical emergency with 10 to 20 percent incidence of early hemorrhagic death due to life-threatening coagulopathy. Patients with AML in general, and APL in particular, generally present with symptoms related to complications of pancytopenia (anemia, neutropenia, and thrombocytopenia), weakness and easy fatigability, infections of variable severity, hemorrhagic findings such as ecchymoses, gingival bleeding, epistaxis, or menorrhagia. Unique to APL is the presentation with bleeding secondary to disseminated intravascular coagulation (Warrell et al., 1993). Disseminated intravascular coagulation (DIC) is frequently present at diagnosis or occurs soon after the initiation of cytotoxic chemotherapy in patients with APL. In children, the disease is often associated with a high white blood cell count (WBC > 10,000/μL), the micro-granular variant (M3V), and more ATRA related toxicities (Testi et al., 2005).

The initial approach to a suspected APL is reviewing the morphology. APL is characterized by the presence of atypical promyelocytes in the bone marrow and peripheral blood. The peripheral blood smear often shows leukopenia with circulating promyelocytes, which usually have abundant, often irregular-appearing primary azurophilic granules. Leukemic promyelocytes with multiple auer rods may be found

and are identified only in APL. Although often obscured by the granules, the nuclear contour is bilobed or reniform in appearance (Tallman and Altman, 2009).

The immunophenotypic (IPT) features of APL blasts shows expression of CD13 and CD33, weak, or negative CD34 expression, and dim to negative expression of HLA-DR and CD11b. The identification of the APL-specific genetic lesion in leukemic cells is feasible at chromosome, DNA, RNA, and protein levels with the use of conventional karyotyping, fluorescence in situ hybridization (FISH), reverse transcriptase polymerase chain reaction (RT-PCR), or anti-PML monoclonal antibodies, respectively. Metaphase karyotyping has the advantage of being highly specific and can detect variant translocations and should be obtained in every patient with suspected APL. Reverse-transcriptase polymerase chain reaction (RT-PCR) for the PML-RARα fusion transcript is also routinely obtained. Florescence in situ hybridization can be carried out but adds little to routine karyotyping and RT-PCR. PML antibody testing is rapid and can be positive in patients with atypical breakpoints (Figure 17).

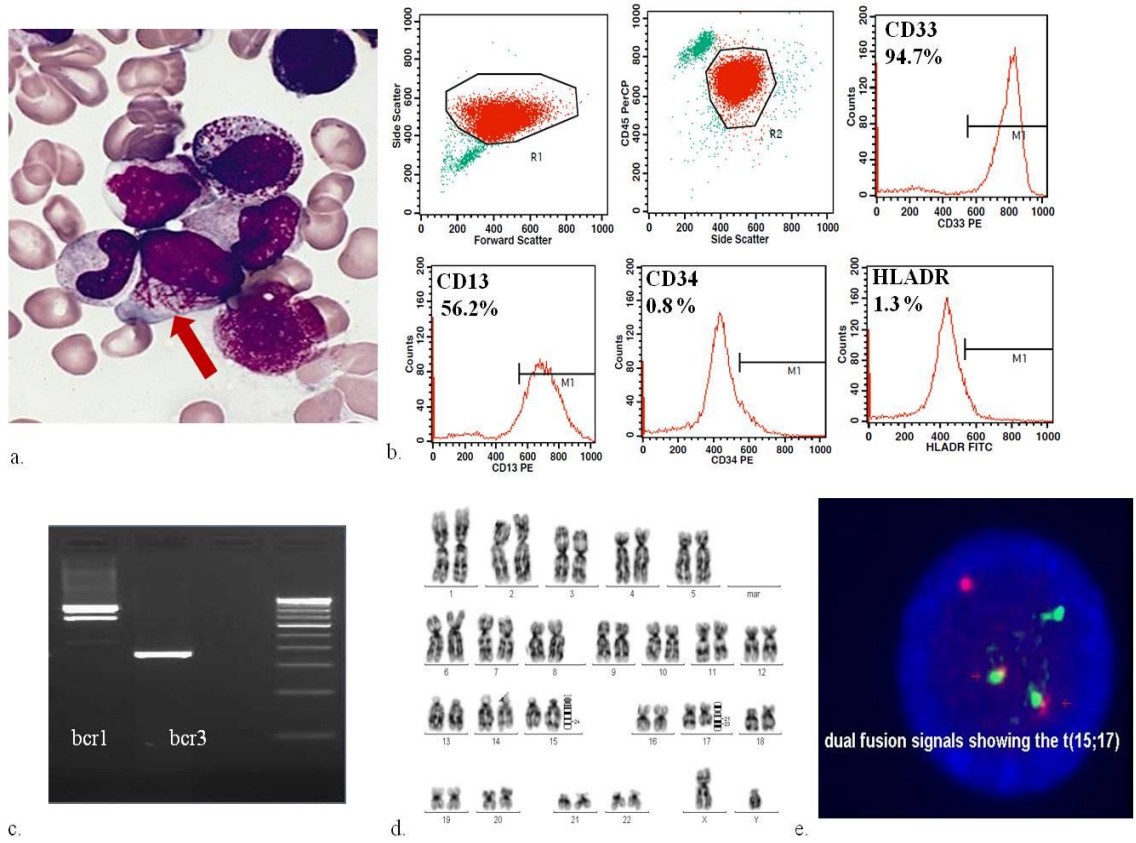


Figure 17. Methods for diagnosis of APL*. a. Morphologic studies require a Wright-Giemsa or May-Grunwald derived stain usually complemented with myeloperoxidase or Sudan black B stain. In the hypergranular type, atypical promyelocytes with a high nucleus to cytoplasmic ratio, fine chromatin and prominent nucleoli, the presence of dense violet granules, folded or bilobed nuclei and have bundles of auer rods in cytoplasm called as 'faggot cells' are seen. In the micro-granular type constituting 25% of cases, the abnormal promyelocytes mostly have bilobed nuclei with very few or complete absence of granules. b. FSC/SSC gating of APL bone marrow sample showing the characteristic CD33 and CD13 positivity and negative for CD34 and HLADR. c. Qualitative nested RT-PCR for PML-RARα showing bcr1 and bcr3 transcripts. d. Karyotyping on G-banded metaphases showing t(15;17) translocation. e. FISH analysis of PML-RARα showing dual fusion signals, two orange signals corresponds to der (15) and der (17) and one green (normal 17 RARα) and one red (normal 15 PML).*

2.7.3. Therapy in APL

APL represents a unique model in cancer biology were two therapeutic agents target PML-RARα through nonoverlapping pathways - retinoic acid and arsenic trioxide. Based on its concentrations both drugs induce differentiation of leukemic cells in varying extents in vivo (Zhu et al., 2001) making APL the first example of successful differentiation therapy of a human cancer.

In 1960s use of chemotherapy in induction along with 6-mercatopurine (6-MP) and other steroids results in poor remission rates of 5-14% with a high incidence of early hemorrhagic death. Chemotherapy was aimed only to inhibit the proliferation of malignant cells. The use of daunorubicin as a mono-chemotherapy was introduced in 1973 by J Bernard and co-workers and showed 55% complete remission (CR) in APL patients (Bernard et al., 1973). Following this landmark study, anthracycline-based therapy (daunorubicin, idarubicin, cytosine arabinoside) became the frontline treatment of APL, with CR rates of 55- 80% in newly diagnosed patients and 5year DFS of 35-45%. The bleeding syndrome observed with chemotherapy demands the use of intensive platelet and fibrinogen support.

The introduction of all-trans retinoic acid (ATRA) in the mid-1980s by the Chinese groups revolutionized the management and outcome of APL. ATRA which is a derivative of vitamin A was reported to induce clinical remissions in APL (Huang et al., 1987). Treatment of APL with ATRA became the first example of a non-cytotoxic

differentiation induction therapy of human cancer and first successful molecular targeted drug. *In vitro* and *in vivo* studies revealed that administration of ATRA at pharmacological concentrations (10^{-6}M) induced morphological maturation of leukemic cells, leading to complete remission. The capacity of retinoid to induce myeloid differentiation was recognized prior to the identification of the involvement of RARα in APL. Single agent, ATRA-induced CR rates of 70-85%, but still the relapses were higher. Two randomized trials of the European APL group and the North-American Intergroup showed that its effect was greatly increased in combination with chemotherapy (Fenaux et al., 1993, Tallman et al., 2002a). Combining ATRA and chemotherapy has further raised the CR rate up to90- 95%, and a 5-year DFS in 50-75% cases (Tallman et al., 2002b). Thus APL which was recognized as a fatal disease in the pre-ATRA period (1957-1985) has become the most curable subtype of adult AML. Incorporation of ATRA also results in rapid resolution of the characteristic life-threatening coagulopathy as it has an impact on the hemostatic system. Still, there were 5-10% early deaths within one month after the start of ATRA treatment with or without chemotherapy. In the early ATRA era, use of ATRA alone induces an elevation of white blood cell (WBC) count resulted in retinoic acid syndrome (RAS) in 6-31% cases, and the incidence was decreased by the early addition of chemotherapy with ATRA.

2.7.3.1. Mechanism of action of ATRA

Pharmacological concentration (10^{6}-10^{7}M) of ATRA causes a configuration change of PML-RARα. Retinoic acid induced APL differentiation are enhanced by

cAMP signaling which activates protein kinase A (PKA), which dissociates RARα from SMRT co-repressor, allowing transcriptional activation through RXRα, whereas a coactivator complex composed of proteins with histone acetylase (HAT) activity is recruited, opening the chromatin structure and relieving transcriptional repression and induce its degradation (Dilworth and Chambon, 2001). Although it was reported that ATRA could trigger caspase-mediated cleavage of the PML-RARα chimeric protein, further dissection of the pathways involved in PML-RARα catabolism led to the discovery of a ubiquitin / proteasomes system (UPS)–mediated degradation of PML-RARα and RARα, which was dependent on the binding of SUG-1 in the AF-2 transactivation domain of RAR.

Arsenic trioxide

The journey of arsenic from poison to therapeutic drug started when Hippocrates first used orpiment (As_2S_3) and realgar (As_2S_2) for ulcer remedy. The arsenical compounds were used as early as 2000 BC, from then, physicians and scientists have broadened the knowledge on this magic bullet. Arsenic has long been known to act as a carcinogen, involved in human skin, lung, liver, kidney and urinary bladder cancers (Huff et al., 2000). Arsenic trioxide (As_2O_3/ATO) was first used in the traditional Chinese medicine. In Western medicine, arsenous oxide (Fowler's solution) was used as a treatment of choice for chronic myeloid leukemia (CML) in the 19^{th} century. However, due to the toxic side effects of long-term high dose of oral arsenic in most patients, and

with the advent of modern radiotherapy and chemotherapy, the arsenic treatment for CML was given up in Western medicine (Kwong and Todd, 1997, Tamm et al., 1999).

Arsenic trioxide (ATO) has been successfully introduced in the treatment of newly diagnosed APL, both as a single agent and in combination with conventional agents. Shen et al. reported the first controlled trial using purified ATO in treating APL and investigated the pharmacokinetics of ATO *in vivo* (Shen et al., 1997). In our center we have been using the single agent ATO for the newly diagnosed APL from 1998. Studies from Shen et al. described the dose of ATO as 10mg a day for adults till complete hematological remission was achieved (Shen et al., 1997). Subsequent studies and our center also used the similar doses of ATO.

ATO is shown to be clinically effective in ATRA-resistant patients (Niu et al., 1999). Though ATO has been shown to be effective in the management of relapsed and newly diagnosed cases of APL, a proportion of newly diagnosed (10-20%) and relapsed cases (30-60%) will eventually relapse following therapy with single agent ATO (Mathews et al., 2002). In our socio-economy status, a single agent ATO based regimen has been used as a less expensive alternative to treat patients with newly diagnosed APL in the setting of a clinical trial (Mathews et al., 2010). ATO is a clinically approved in the treatment of APL both as a single agent as well as in combination with all-trans retinoic acid (ATRA) (Powell et al., 2010). This is a targeted therapy as recent studies have demonstrated direct interactions between ATO and the PML moiety of the PML/RARα oncoprotein, (Jeanne et al., 2010, Zhang et al., 2010). Using single-agent

ATO, it is noted that detection of PML-RARα at the end of the initial induction therapy correlates best with subsequent risk of relapse (Chendamarai et al., 2012). Data from our center showed that treatment with an ATO-based induction and consolidation followed by an autologous SCT in molecular remission could achieve a cure in up to 70% of cases, even after hematologic relapse.(Thirugnanam et al., 2009).

Besides the significant clinical effectiveness of ATO for patients with APL, acquired resistance to this therapy has been recognized in clinical practice. A recent study by Goto et al. showed that the patients who showed clinical ATO resistance harbored missense mutations in PML-RARα transcripts, resulting in amino acid substitutions of A216V and L218P in the PML B2 domain critical for PML localization and ATO responsiveness *in vitro* (Goto et al., 2011).

2.7.3.2. Mechanism of action of ATO

Even though the mechanisms by which ATO induce remission is not clear, some of its cellular mechanisms are studied. ATO exerts dose-dependent dual effects on APL cell. Under high concentrations (1-2x10^6M), ATO induces apoptosis, mainly through activating the mitochondria-mediated intrinsic apoptotic pathway. Under low concentrations (0.25-0.5x10^6M) and with a longer treatment course, ATO tends to promote differentiation of APL cells (Chen et al., 1997). ATO is a drug with minimal myelosuppression. ATO appears to act in APL via multiple mechanisms, and the key mechanisms appear to be via PML-RARα degradation, arsenic-induced oxidative stress,

loss of mitochondrial membrane potential, caspases release and activation eventually leading to apoptosis (Chen et al., 1996, Shao et al., 1998). The *in vitro* studies in APL cell line NB4 showed that ATO induces apoptosis by down-regulation of Bcl-2 (Chen et al., 1996).

Arsenic is known to interact with proteins rich in cysteine residues and accessible thiol groups. The mechanism by which this agent cures this disease has previously been shown to involve direct interactions between ATO and the promyelocytic leukemia protein (PML) as well as accelerated degradation of the APL-associated fusion oncoprotein PML- RARα by ROS-mediated PML oxidation (Jeanne et al., 2010). Another study shows that ATO causes the stabilization of nucleoporins (CyPN) with inhibition of nuclear PML body formation after mitosis, suggesting that this drug blocks the cell-cycle–dependent PML body circuit at the level of nuclear import (Lang et al., 2012). It has been shown that PML-RARα degradation is tightly regulated by a PKA phosphorylation site which in turn triggers LIC loss, is responsible for eradicating APL in mice (Nasr et al., 2008). Two groups reported that ATO binds directly to cysteine residues in zinc fingers of located within the PML RBCC motif that contains three cysteine-rich zinc-binding domains, a RING-finger (R), two B-box motifs (B1 and B2), and a coiled-coil (CC) domain, in PML-RARα and PML (Zhang et al., 2010, Jeanne et al., 2010). This results in oligomerization of PML-RARα or PML and promotes SUMOylation leading to ubiquitination and degradation of PML and PML-RARα oncoprotein.

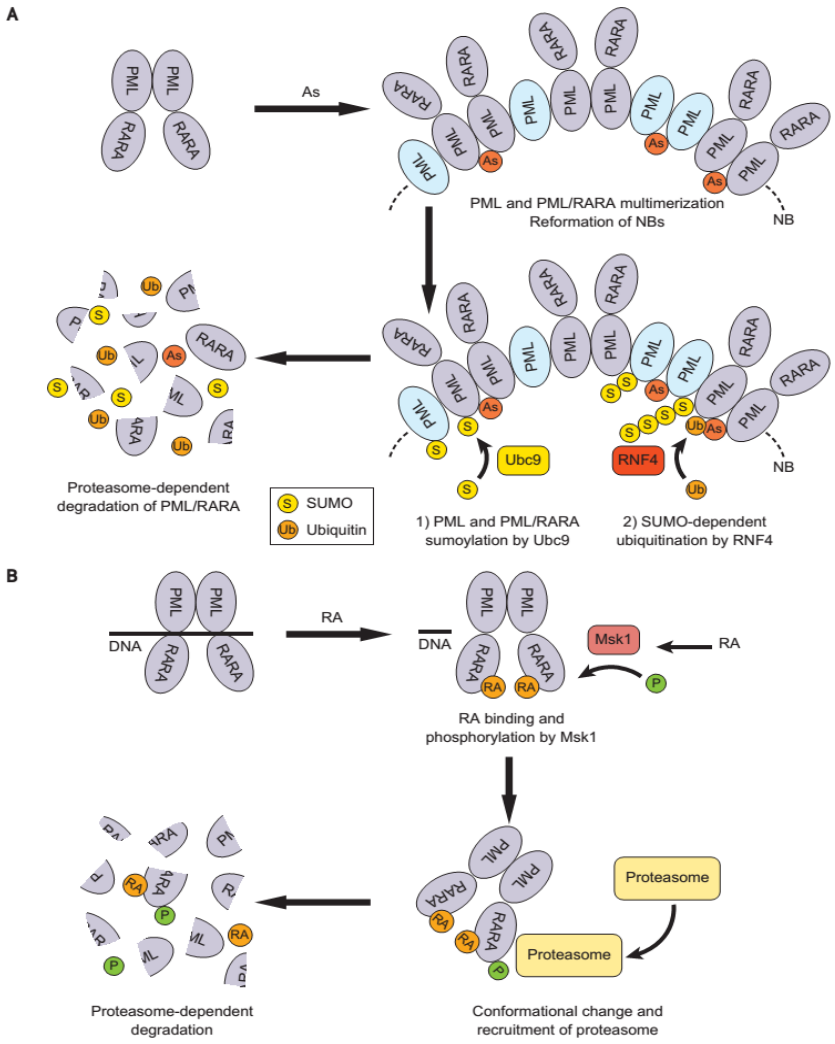


Figure 18. Therapy-induced degradation of PML/RARα. *A. Mechanism of arsenic-induced PML/RARα degradation. Arsenic directly bridges PML/ RARα proteins 2 by 2, which allows the formation of nuclear bodies (NB) in which the PML moiety of PML/RARα is first sumoylated by the Ubc9 conjugation enzyme and then polyubiquitinated by the SUMO-dependant E3 ligase RNF4, resulting in PML/RARα proteasome-dependent degradation. B. Mechanism of RA-induced PML/RARα degradation. RA docks to the ligand-binding domain of the RARα moiety of PML/RARα, triggering a conformation change that is also favored by RA-induced RARα phosphorylation by Msk1. The conformation change allows subsequent recruitment of the 26S proteasome, resulting in PML/RARα degradation. Adapted from (Ablain et al., 2011)*

2.8. Mouse model of APL

'*Nature has been generous to science and has provided us with many model systems. How the great diversity of the living world can both inspire and serve innovation in biological research'.*

- *Nature's gift to Science, Nobel Lecture, December 8, 2002 by Sydney Brenner*.

Laboratory mice are the most experimentally accessible mammalian model for understanding and conceptualizing the complexity of human cancer pathogenesis and to develop improved anti-tumor therapies. It shares organ systems, systemic physiology and genes with humans and the main highlight is their ease in using for genetic manipulation. When a PML-RARα cDNA derived from t(15;17) translocation is placed under control of human cathepsin G regulatory sequences, it is expressed in early myeloid cells at low levels. Virtually all mice expressing PML-RARα in early myeloid cells develop a myeloproliferative syndrome, and 15% to 20% go on to develop a disease that closely resembles acute promyelocytic leukemia after a latent period of 6 to 14 months (Grisolano et al., 1997).

To establish a knock-in model with high penetrance, they targeted human PML-RARα cDNA to the 5' untranslated region of the murine cathepsin G gene, using homologous recombination in embryonic stem cells. This model produced a high-penetrance APL phenotype, with more than 90% of knock-in mice developing APL between 6 and 16 months of age (Westervelt et al., 2003). However, the expression level of PML-RARα in bone marrow cells or APL cells was less than 3% in the low

penetrance transgenic model. The penetrance of this APL-like disease can be increased by nearly 4-fold by coexpressing the reciprocal RARα-PML cDNA in the same early myeloid compartment, thus the long latency persists in these transgenic mice (Pollock et al., 1999) suggesting that PML-RARα is the primary determinant of the phenotype of this disease and that it is a bonafide leukemia-initiating protein (Pollock et al., 2001). Other investigators have created transgenic mice were PML RARα is expressed under CD11b gene, but this model failed to develop either pre-leukemia or leukemia (Early et al., 1996). The transgenic mice described by Brown et al using the human PML-RARα cDNA, cloned from a patient with a PML-RARα bcr1 break point, was constructed in the FVB/N inbred strain of mice with the hMRP8 promoter. (Brown et al., 1997). This murine leukemic model was a true analog to human APL in terms of recapitulating the disease and differentiation in response to ATRA.

2.9. Immune response in APL

The cells from the spleen of an APL transgenic mouse are transplanted into syngeneic recipients. This model mimics the human APL in its biological characteristics and its response to conventional therapeutic drugs such as ATRA or ATO. Westervelt and his group in 2002 have shown that adaptive immunity cooperates with liposomal ATRA to facilitate long-term molecular remissions in mice with acute promyelocytic leukemia. They used an adoptive transfer model of murine APL to investigate the efficacy of ATRA in immunocompetent and immunodeficient mice. Their observations suggest that the B and T cells of naive immunocompetent mice must be capable of

mounting effective tumor-specific responses against APL cells and that these responses facilitate durable remissions on treatment with ATRA than the SCID (severely combined immunodeficiency) mice (Westervelt et al., 2002). Studies have shown that ATRA therapy by itself can trigger an immune response in this mouse model. Studies have shown that ATRA in combination with a vaccine of specific tumor associated antigen DNA fused to tetanus fragment C has a pronounced survival advantage with time-dependent antibody production and an increase in IFNγ (Padua et al., 2003). This model could provide a new approach to improve clinical outcome in human leukemia. It is currently well recognized that in mice and in patients with APL, antibodies against RARα and myeloid proteins are frequently present and potentially contribute to the maintenance of sustained molecular remission (Robin et al., 2006). Also, recent studies from the same group in collaboration with our center have shown the advantage of pVAX14 vaccine along with ATO+ATRA (Patel et al., 2015).

Despite its rarity, APL is a key model to study the mechanisms of leukemogenesis and a successful example for translational research in medicine as a tool for innovative treatments. The use of ATRA and ATO frontline had lead to extremely high rates of cure and improved the quality of life of the patient. Even though the efficacy of ATO in the management of APL is undebatable, the concerns regarding relapses and mechanisms to enhance its efficacy have to addressed in detail. With the establishment of animal models and cell-based models of APL, more effective studies can be done.

MATERIALS AND METHODS

3.1. Research design

This study aimed to evaluate the impact of ATO on the immune and cellular response in acute promyelocytic leukemia patients treated with ATO and in APL mouse model. It is believed that the immune response plays a significant role in preventing relapse and has been demonstrated that immune response is important in sustaining long-term molecular remission in a transplantable mouse model of APL. The available data suggests that the immune response co-operates with therapy to ensure cure in this malignancy and this needs to be studied systematically. The impact of ATO on innate and adaptive immune system is not well studied and the immune subset recovery and reconstitution pattern post chemotherapy has never been evaluated in patients with APL treated with ATO. Towards this, we comprehensively evaluated the role of ATO on immune subset reconstitution and its impact on immunomodulation in mice and patients. We also attempted to study the efficacy of DNA vaccine as an adjuvant to ATO in APL mouse model. Understanding factors that predict clinical outcomes is critical to further improve the efficacy of this therapy.

The following were the major experiments done with cell lines, peripheral blood and bone marrow mononuclear cells from APL patients and in APL mouse model.

3.1.1. Immune reconstitution studies in APL patients: The immune reconstitution pattern in newly diagnosed APL patients were studied at different time points by flow cytometry

analysis. The differential reconstitution pattern of immune subsets was studied and was correlated with clinical outcomes.

3.1.2. Characterization of immunosuppressive myeloid-derived suppressor cells (MDSC) subsets: Peripheral blood mononuclear cells were evaluated for the presence of immune suppressive MDSCs phenotypically defined as CD14-HLADR-CD33+CD11b+ and was correlated with clinical outcomes.

3.1.3. Evaluation of antibody response in APL patients: Serum samples collected from APL patients was screened for the presence of anti-RARα antibodies by ELISA, and the specificity of anti-RARα antibodies was confirmed by immunoblotting.

3.1.4. In vitro cytotoxicity assay for ATO sensitivity: The *in vitro* cytotoxicity of ATO towards myeloid and lymphoid cell lines, NK92MI cell line and PBMNCs were tested by MTT assay and IC-50 was calculated.

3.1.5. Evaluating the effect of arsenic trioxide on the modulation of immune cells: The effect of ATO on the proliferation and its role in the cytolytic activity of NK cells was evaluated by flow cytometry by CFSE/7AAD cytotoxicity assay and degranulation assay. Expression profile of NK ligands and receptors induced by ATO was also evaluated.

3.1.6. NK cell receptor genotyping: The presence or absence of activating and inhibitory KIR genes were done using the genomic DNA isolated from APL patients and haplotype analysis was done.

3.1.7. Assessment of NK differentiation from CD34: CD34 positive cells were sorted from cord blood sample, and NK cells were differentiated with and without ATO treatment and assessed on day 8 and day14. The role of NK transcription factors in NK differentiation were studied by RQ-PCR.

3.1.8. Establishment of the preclinical animal model of APL treated by arsenic trioxide: The transplantable APL mice model was established and different treatment interventions were done. Mice were monitored for blood counts, organ pathology, survival and other parameters. The safety and efficacy of DNA vaccine as an adjuvant to ATO in this mouse model were assessed.

3.1.9. Cellular therapy by NK and IL-15 in APL transplantable mouse model: FVB/N mice treated with ATO or ATRA or in combination was given NK cells (sorted from FVB/N spleen) with or without IL-15 (100ng /mouse given in 5 doses) and was assessed for survival.

The work flow of the experiments in APL patients, cell lines and in APL mouse model is outlined in Figure 19.

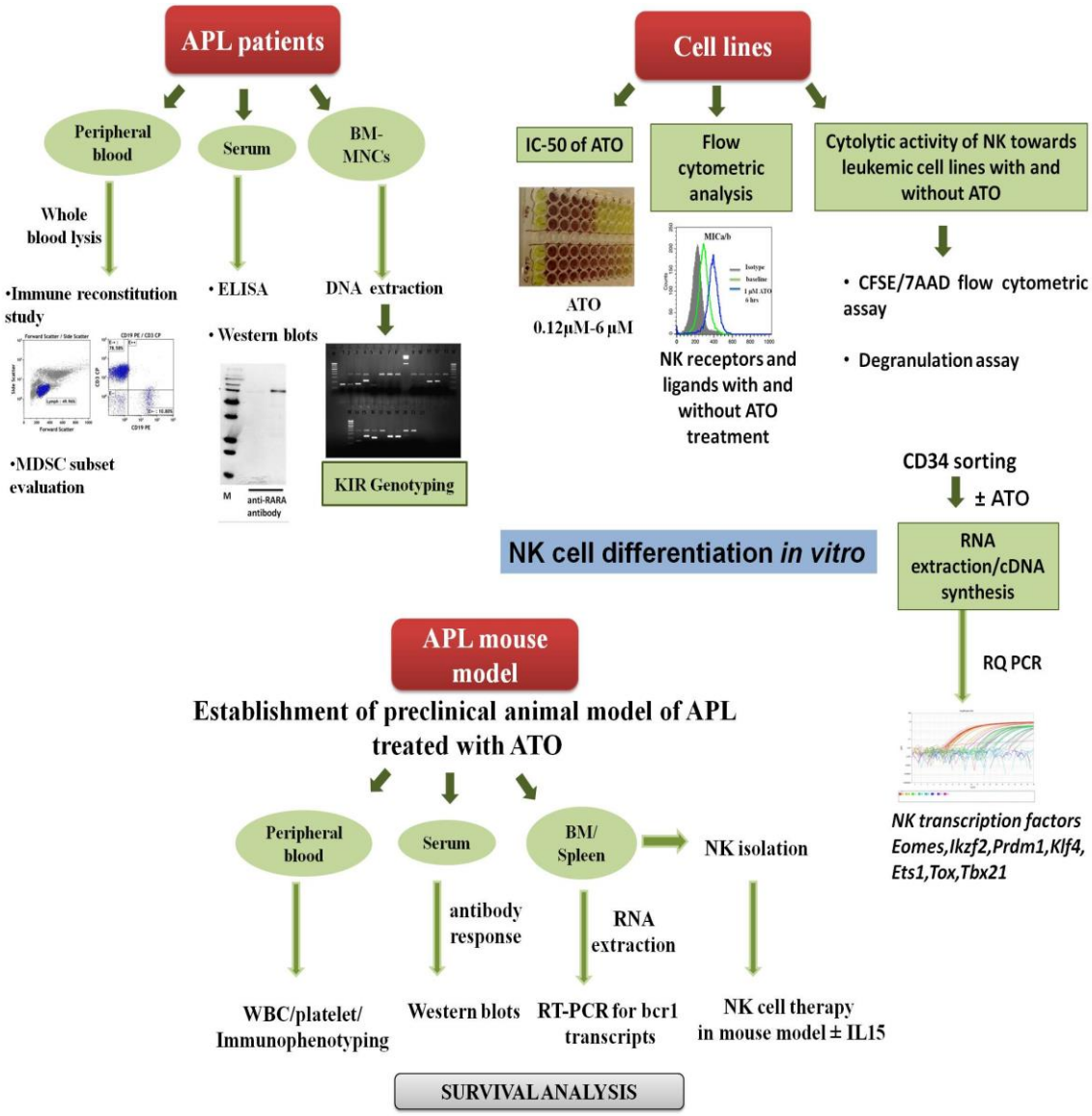


Figure 19. Flow chart summarizing the study "Evaluation of immune response in acute promyelocytic leukemia treated with arsenic trioxide."

3.2. Cell lines

The human APL cell lines used in this study were NB4 (kind gift from Dr. Harry Iland, RPAH, Sydney, Australia with permission from Dr. Michel Lanotte), UF1(kind gift from Pr. Christine Chomienne Inserm UMR-S940 Institut Univérsitaire d'Hématologie, Hôpital Saint-Louis, Paris) and an in-house generated ATO-resistant cell line NB4EVAsR1. NB4 cells in our laboratory were exposed to serial increasing concentrations of ATO over a period of 1 year and when stable at 1µm concentration of ATO, clonal populations was further isolated by limiting dilutions and methylcellulose plating to generate NB4EVAsR1(unpublished data). All the other cell lines were sourced from American Type Culture Collection (ATCC, Rockville, MD, USA). The cell lines were cultured at 37ºC in a humidified atmosphere containing 5% CO2 and were characterized phenotypically by flow cytometry and are free of mycoplasma contamination (Universal Mycoplasma detection Kit, ATCC) and documented. Table 2 shows the different cell lines used in this study.

3.3. Plasmids

pVAX14 (pVAX14 flipper) DNA plasmid vaccine, the plasmids PGEX2T-GST and PGEX-2T full-length RARα were obtained as a kind gift from Pr. Christine Chomienne, INSERM, UMR-S940, Paris.

The chemicals and reagents used in the study are mentioned in Appendix A-1

Table 2. List of different cell lines used in this study, type of cell line, its source and the media used for culture.

Cell line	Type	Source	Culture media
NB4	promyelocytic	Dr. Harry Iland, RPAH, Sydney, Australia	10% RPMI
UF1	promyelocytic	Pr.Christine Chomienne, Hôpital Saint-Louis, Paris	15% RPMI
K562	myeloid	ATCC	10% DMEM
HL60	myeloid	ATCC	20% RPMI
U937	myeloid	ATCC	10%RPMI
Jurkat E6.1	lymphoid	ATCC	10%RPMI
SUP-B15	lymphoid	ATCC	20%IMDM with supplements
NK92MI	NK	ATCC	MEM with 0.2mM inositol, 0.1mM 2-ME, 0.02mM folic acid, 12.5% horse serum and 12.5% FBS

3.4. Study patients

Participants and setting: Patients newly diagnosed with acute promyelocytic leukemia from March 2010- May 2015 treated with single agent ATO which is an ongoing clinical protocol in the Department of Hematology, Christian Medical College, Vellore were enrolled in this study after getting written and informed consent (consent form attached Appendix A-2). The study was approved by the Institutional Review Board, Christian Medical College, Vellore, Tamil Nadu (IRB Min no: 7081 dated 17.02.2010).

Inclusion criteria: To be included in the study APL patients were diagnosed on morphology based on FAB criteria and the presence of t(15;17) translocation was confirmed either by Fluorescent in-situ hybridization (FISH) or reverse-transcriptase-polymerase chain reaction (RT-PCR) looking for PML-RARα transcripts.

Exclusion criteria: Samples were excluded from the study if there is a failure to confirm the APL diagnosis by molecular methods. Patients who have received treatment elsewhere were also excluded.

Treatment Protocol: The clinical trial using ATO as a single agent was initiated in January 1998 at our institution and ATO was prepared in the pharmacy department of this institution. From December 2003, commercially available ATO was utilized (INTAS Pharmaceuticals Ltd, Matoda, Gujarat, India). 10 mg per 10ml ATO vial was diluted in 500 ml dextrose saline and administered for adults and dilution in 200 ml dextrose saline for pediatric population and was infused intravenously over 2 to 3 hours, once a day, without electrocardiographic monitoring. The schedule of single-agent ATO used in this study is summarized in Figure 20. Supportive care and monitoring were done (Mathews et al., 2006).

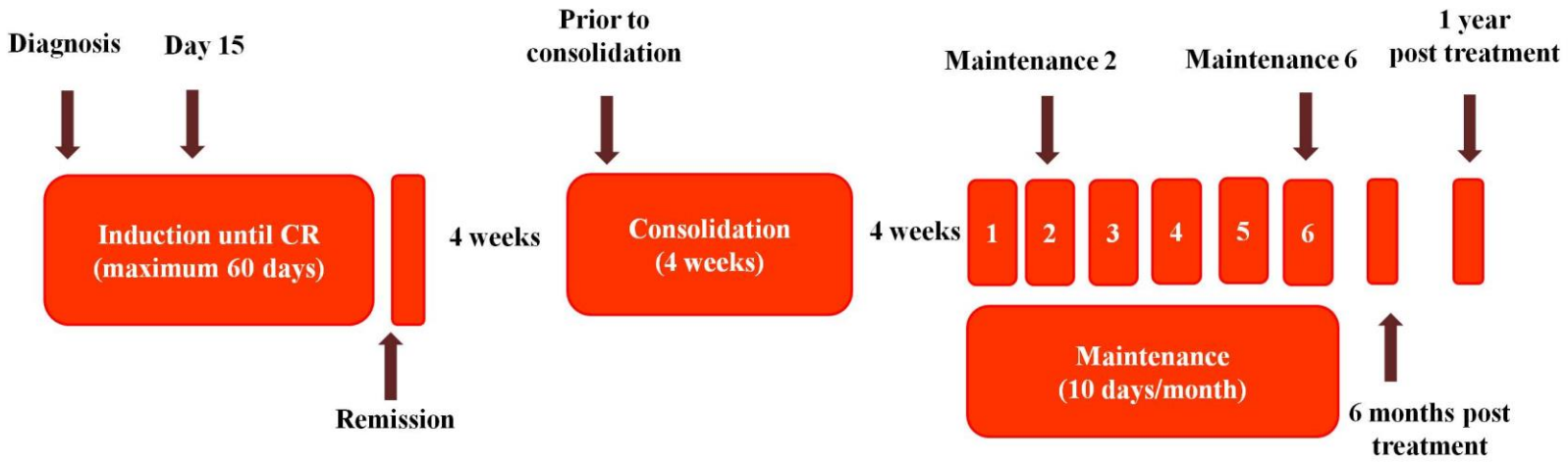


Figure 20. Diagram showing the treatment protocol followed in our institution for newly diagnosed APL and time points of sample collection for this study*. Arsenic trioxide was administered intravenously at a dose of 10 mg for adults and 0.15 mg/kg for pediatric patients. Single-agent ATO was administered until complete hematologic remission (CR) for a maximum of 60 days. Following a 4-week interval, single agent ATO was administered for another four weeks as a consolidation course, for those in CR. Again after a 4-week interval, for those continuing to remain in CR, single agent ATO was administered ten days a month for six months (Mathews et al., 2006).*

Definition of outcomes: For achieving complete remission (CR) patients should not have any clinical evidence of APL, an absolute neutrophil count (ANC) higher than 1.5 x 10^9/L, an unsupported platelet count of more than 100 x10^9/L, and a bone marrow analysis showing normocellularity to moderate hypocellularity with less than 5% blasts plus promyelocytes. Exception was made in patients who achieved unsupported platelet count higher than 100 x10^9/L for more than 2 weeks with no evidence of residual disease as defined previously but had an ANC lower than 1.5 x 10^9/L (Mathews et al., 2010). Molecular relapse was defined as two consecutive positive RT-PCRs at an interval of 1 month apart after achieving molecular remission. Overall survival (OS) was defined as the time from initiating treatment to last follow-up or death. Event-free survival was calculated from the time of initiating therapy to last follow-up or an event (relapse or death). Disease-free survival (DFS) was calculated from the time of achieving CR to last follow-up or an event (relapse).

3.4.1. Sample collection

Peripheral blood (PB) or bone marrow (BM) samples (5-6ml) were collected from APL patients at diagnosis. Sample from each patient were given a Unique Patient Number (UPN). Samples were collected in sterile K_3EDTA vacutainer (Greiner Bio-One International, GmbH, Austria) for DNA and RNA studies. Sodium heparin tubes (Greiner Bio-One International, GmbH, Austria) were used to collect PB for immune subset analysis. Serum samples were collected from clotted red vacutainer tubes

(Greiner Bio-One International, GmbH, Austria). The samples were collected by the nursing staff in our department.

Time points of sample collection

Peripheral blood and serum samples are collected at the following time points (Figure 20).

(i) pre-treatment sample

(ii) sample after initiating therapy on day 15

(iii) at the time of documentation of remission (~day 40-60)

(iv) prior to onset of consolidation

(v) maintenance therapy cycle 2

(vi) maintenance therapy cycle 6

(vii) 6 months post treatment

(viii) one year post treatment

3.4.2. Sample processing

Serum was separated by centrifuging at a speed of 2500 rpm for 15 minutes and was snap freezed at -80^{o}C. Mononuclear cells (MNCs) were isolated using Ficoll-paque density gradient centrifugation (GE Healthcare, Foster City, California, USA) and used for ex-vivo cytotoxicity and gene expression studies. DNA was extracted using Gentra Puregene Blood kit (Qiagen, Hilden, Germany) for genotyping studies and was stored at 4^{o}C. RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Massachusetts,

United States) and stored at -80ºC for quantitative real-time PCR experiments. PB samples collected from healthy volunteers were used as controls and were stored as mentioned above.

3.4.3 Morphological analysis

Bone marrow biopsy slides of all newly diagnosed and relapsed APL patients stained with May-Grünwald-Giemsa stain (MGG) were reviewed prior to the starting of treatment by the hematopathologists at our center. MGG stain is a modified Romanowsky stain which combines the effect of acidic eosin, azure and alkaline methylene blue which selectively stains the tissues. MGG stains nucleus purple and cytoplasm blue/pink.

3.4.4. Molecular diagnosis by Qualitative Reverse Transcriptase PCR (RTPCR)

The PML-RARα fusion gene transcripts bcr1, bcr2, and bcr3 types were amplified by a 2-step (nested) qualitative RT-PCR. This was done using the recommendations of BIOMED-1 Concerted Action (van Dongen et al., 1999). A volume of 2µl of cDNA was diluted into 25µl of a PCR mixture containing 400µM primers, 200µM dNTPs, PCR buffer and 0.2U of AmpliTaqTM polymerase. PCR cycles included an initial denaturation at 95^0 C for 10 minutes. Melting, annealing and extension were carried out at 95^0 C for 30 sec, 65^0 C for 1 min, and 72^0 C for 1 min, respectively, for a total of 35 cycles. Nested PCR was performed under same conditions, but using 0.75µl of PCR product from the first round and internal primers. Finally, 10µl of the PCR

products were analyzed in a 2.5% agarose gel stained with ethidium bromide and visualized under UV light.

3.4.5. Flowcytometric immunophenotyping

Direct Immunofluorescence staining of single-cell suspensions was performed for detection of surface and intracellular antigens. The titrated volume of monoclonal antibodies directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), allophycocyanin (APC) (Becton Dickinson, San Jose, CA, USA) as listed in Table 3, were dispensed into each of the tubes containing patient WBCs and incubated for 20 minutes at room temperature in the dark. After washing (PBS-AZIDE with 0.1% BSA), the cells were resuspended again in 200µl of wash buffer and acquired on FACS Calibur (Becton Dickinson, Mansfield, MA, USA). A total of 20,000 events were acquired, and the data was analyzed using BD CellQuest Pro software (Becton Dickinson, Mansfield, MA, USA). Results were expressed as percentage positivity of gated abnormal population (Figure 21).

Table 3. Panel of monoclonal antibodies used for APL diagnosis by flow cytometry *(courtesy: Flow cytometry lab, Department of Hematology)*

AL 45 Panel for Acute leukemia

	FITC	PE	PerCP	APC
Tube 1	IgG1	IgG1	CD45	IgG1
Tube 2	CD7	CD5	CD45	CD3
Tube 3	CD64	CD34	CD45	CD117
Tube 4	CD19	CD10	CD45	CD20
Tube 5	HLADR	CD33	CD45	CD11b
Tube 6	CD4	CD56	CD45	CD13

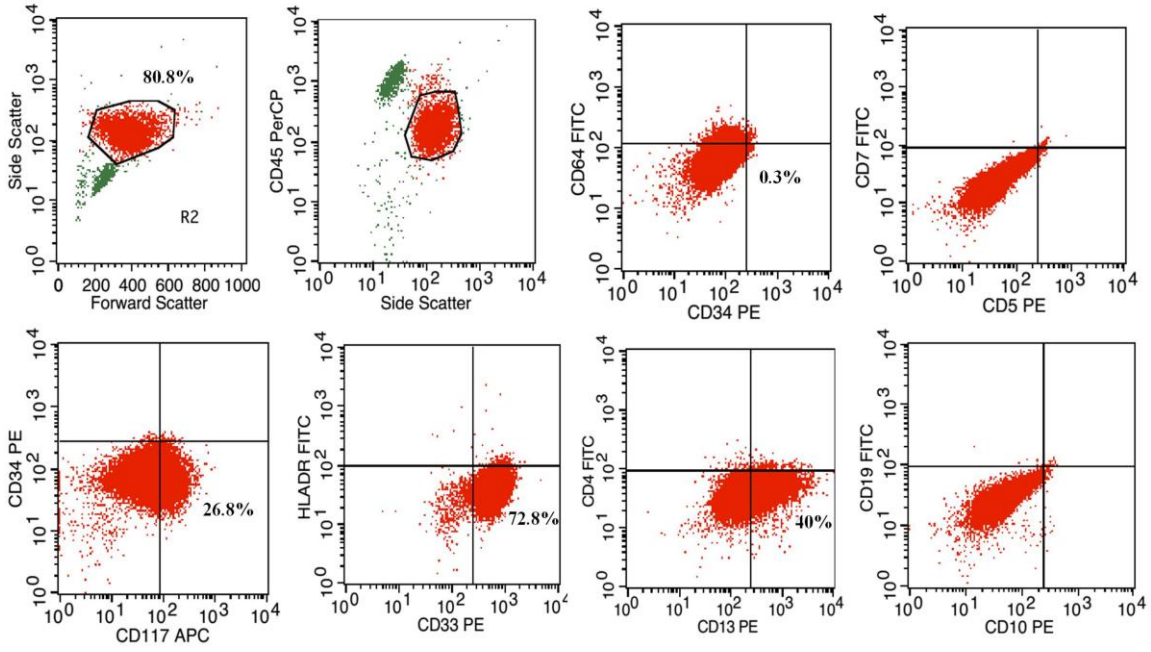


Figure 21. Representative dot plots showing the classical immunophenotype of APL. *The CD45 dim population is positive for the myeloid markers CD13, CD33 and CD117 and negative for CD34 and HLADR. T cell (CD7 and CD5) and B cell specific markers (CD19 and CD10) were absent.*

3.4.6. Cytogenetical Analysis

All cytogenetic studies were performed at the cytogenetics unit of CMC according to standard protocols with the karyotypes designated according to the International System for Human Cytogenetic Nomenclature (ISCN) guidelines. Briefly, fresh bone marrow samples, collected in sterile sodium heparin vacutainers were used for karyotype analysis. 1.5×10^6cells /ml cells are seeded in RPMI 1640 with 20% FBS and cultured overnight. To one set of cultures, 0.02µg/ml of colcemid was added and incubated overnight. To another set of cultures, 0.05µg/ml of colcemid was added and incubated for 1 hour. Cultures were then harvested, and slides were made and banded. Images were analyzed using Ikaros software (Metasystems, Germany). At least 20 metaphases were analyzed from each patient, whenever possible. An abnormality was considered to be clonal if ≥ two metaphases showed the same additional chromosome(s) / the same structural abnormality or ≥ three metaphases showed a loss of the same chromosome (Shaffer et al., 2013). Based on the karyotype, patients were allocated into different groups, namely those with an isolated t(15;17) and those with t(15;17) and an additional CTG abnormality. Patients with karyotypes showing >2 abnormalities apart from the t(15;17) were considered to have complex karyotype.

3.5. Isolation of Mononuclear Cells

Density gradient centrifugation by Ficoll-Paque (GE Healthcare, Foster City, California, USA) was used for the isolation of bone marrow mononuclear cells

(BMMNCs) for all the downstream experiments such as DNA and RNA studies. Briefly, bone marrow aspirate (diluted 2-3 times if the counts are too high) after being passed through a 100µm filter for removing bone fragments and cell clumps was carefully layered over Ficoll-Paque solution (2:1 ratio) in a 15ml falcon tube. This was then centrifuged at 400g for 30 minutes at room temperature. The upper plasma layer was aspirated out after centrifugation leaving the interphase layer containing BMMNCs undisturbed. The BMMNC layer was then transferred to a fresh falcon tube, washed and resuspended either in media or PBS and was taken for subsequent experiments.

3.6. DNA Extraction

Genomic DNA was extracted from BM/PB of APL patients. Briefly, red blood cell lysis solution was added (1:5 ratio) to the peripheral blood sample and incubated on ice for 10 minutes and was then centrifuged at 4000 rpm at 4°C for 5 min, and DNA was extracted from the pelleted white blood cells. To the cell pellet, 50-150µl cell lysis solution, (Gentra Puregene Blood kit, Qiagen, Hilden, Germany) was added and incubated overnight at 37°C. After incubation, protein precipitation solution was added (3:1 ratio), vortexed and centrifuged at 4500 rpm for 5 minutes. To the supernatant, an equal volume of isopropanol was added and gently mixed. The DNA was transferred into another tube and washed with 75% ethanol. The tube was then centrifuged at 4000 rpm for 10 minutes after which the ethanol was discarded, and the pellet was air dried. To the pellet, 30-50µl of TE buffer was added and incubated at 55°C till the DNA dissolved completely. The isolated DNA was quantitated using NanoDrop^{R}ND-1000

spectrophotometer (Thermo Scientific, Massachusetts, USA) which measures the absorbance of UV light at 260nm from 1µl aliquot of extracted DNA providing the DNA concentration in ng/µl. A260/A280 value of ~1.8 is the acceptable range for good quality DNA. The DNA was diluted at a concentration of 100ng/µl in TE-Buffer or hydration buffer and stored at 4°C for use in PCR reactions. Genomic DNA extracted from healthy volunteers and patients was used for KIR genotyping (details mentioned in section 3.12.4).

Agarose Gel Electrophoresis

After the PCR reaction, the samples were loaded on a 2% agarose gel. Agarose was dissolved in 1x TBE buffer to an appropriate concentration. Ethidium bromide solution (Bio-Rad, Hercules, CA) was added to a concentration of 0.5 µg/ml before the gel was poured into a casting tray. 10 µl of PCR amplification products were mixed with 5 µl of DNA gel loading dye and then the mixture was loaded into the wells of the gel. 1-kb and 100-bp DNA ladders (Promega, Wisconsin, United States) were used as the molecular weight markers. Electrophoresis was carried out at a constant voltage of 120V in 1xTBE buffer for about 30-50 minutes. A sufficient resolution is ensured by a long gel run to support a clear differentiation of amplicons and was then visualized under ultraviolet (UV) illumination (Gel doc XR Gel documentation system, Bio-Rad, CA, USA) with an UV illuminator and photographed on a camera.

3.7. RNA extraction

Total RNA was extracted from PB/BM-MNCs/cell lines using TRIzol Reagent (Thermo Fisher Scientific, Massachusetts, United States) as per the manufacturer's protocol. Tri Reagent is a mixture of guanidine thiocyanate and phenol in a monophase solution, effectively dissolves DNA, RNA, and protein on homogenization or lysis of tissue sample. Briefly, 2 x 10^7 mononuclear cells were lysed in 1ml of Trizol Reagent (lysed samples can be stored at -80^{o}C for 2-3 months). 0.2ml of chloroform was added per 1ml of Tri Reagent, mixed well and incubated for 8-10 minutes after which the mixture was centrifuged at 12,000g for 15 minutes (2-8^o C). Centrifugation separates the mixture into 3 phases: a red organic phase (containing protein), an inter-phase (containing DNA), and a colorless upper aqueous phase (containing RNA). Aqueous phase was carefully transferred to a fresh tube and to which 0.5 ml of 2-propanol was added and incubated for 10 minutes, centrifuged for 5 minutes at 12,000g (2-8^0 C) so that RNA is precipitated. RNA pellet formed was gently washed with 70% ethanol, centrifuged briefly at 12,000g (2-8^0 C). The RNA pellet was air dried for 2-3 minutes (make sure all the residual ethanol is removed) and 40-60µl RNase free H_2O was added depending on the size of the RNA pellet. For long term storage, store the extracted RNA at -80^0 C.

3.7.1. Assessing quantity and quality of extracted RNA

The concentration of the extracted RNA was determined by NanoDrop® ND-1000 UV (Thermo Scientific, Massachusetts, USA) which measures the absorbance at

260 nm. RNA has its absorption maximum at 260 nm and the ratio of the absorbance at 260 and 280 nm was used to assess the RNA purity. Pure RNA has an A260/A280 of 2. RNA quality was assessed in all samples by running RNA samples in 1% non-denaturing agarose gel in TAE buffer. RNA should appear as two bright discrete bands that represent the 28S and 18S ribosomal species. The 28S band should be brighter than the 18S band. Tailing of these major bands down the gel, or a background smear behind these bands indicate degradation of the RNA. Samples failing quality check are discarded and not considered for further experiments.

3.7.2. cDNA synthesis

cDNA synthesis was performed with 500ng of total RNA using SuperScript® II Reverse Transcriptase (RT) (Invitrogen, CA, USA) following the manufacturer's instructions. For the first strand synthesis reaction, random hexamers were used, which are short oligodeoxyribonucleotides of random sequence that anneal to random complementary sites on RNA and serves as primers for reverse transcriptase. PCR-based cDNA synthesis was performed for all samples following manufacturer's instructions and is stored at -20°C for further downstream applications.

Quality control assessment- post cDNA synthesis

To compensate for potential variability introduced in cDNA synthesis, the expression of housekeeping gene β-glucuronidase (GUS) was assessed by RT-PCR.

GUS has been reported to have the lowest inter-sample variability. Proper amplification of GAPDH by RQ- PCR will also validate the quality of cDNA synthesis.

3.8. Immune reconstitution in newly diagnosed APL patients

To evaluate the pattern of immune subset reconstitution in patients with newly diagnosed APL treated at our center, peripheral blood samples were obtained from patients before treatment, on day 15 after starting ATO, post induction, pre-consolidation, maintenance cycle 2 (6 months from diagnosis), maintenance cycle 6 (10 months from diagnosis), 6 months and 1 year post treatment for flow cytometry analysis. Peripheral blood samples were collected from the patients at the above mentioned time points and are assessed for the following cellular subsets. All the following subsets analysis were done prospectively.

- T cells $CD3^+$
- Helper T cells $CD4^+$
- Cytotoxic T cells $CD8^+$
- Naïve $CD4^+CD45RA^+$
- Memory $CD4^+CD45RO^+$
- Naïve $CD8^+CD45RA^+$
- Memory $CD8^+CD45RO^+$
- B cells $CD19^+$
- NK $CD3^-CD56^+CD16^+$ and its subsets
- Activated Helper T cells $CD4^+CD25^+$
- Dendritic cells - Monocytoid (MC) - $Lin^-HLA\text{-}DR^+CD11c^+$
- Plasmacytoid (PC) - $Lin^-HLA\text{-}DR^+CD123^+$.

Principle

To detect cells bearing specific membrane antigens, whole blood was added to fluorochrome-conjugated monoclonal antibodies, and they bind specifically to cell surface antigens. The stained sample was then added with RBC lysing solution to lyse erythrocytes under gentle hypotonic conditions while preserving the leukocytes. The sample was then washed with PBS to remove excess antibody and debris and analyzed the cells by flow cytometry.

Lysing and Staining

1. Titrated volume of fluorochrome-conjugated monoclonal antibody/antibody cocktail (Table 4) was added to 100 µl of whole blood in a 12 x 75-mm FACS tube.

2. The tubes were then vortexed gently and incubated for 20 to 30 minutes in the dark at room temperature (20° to 25°C).

3. 2 ml of RBC lysis solution was added and vortexed gently and incubated for 10 minutes in the dark at room temperature.

4. After incubation, the tubes were centrifuged at 500g for 5 minutes, and the supernatant was removed.

5. The pellet is then washed with wash buffer (1xPBS with 1% HSA and 0.1% sodium azide) by centrifuging at 500 x g for 5 minutes. The cell pellet is resuspended in 250µl of buffer and was kept at 4°C for acquisition.

Table 4. Panel of monoclonal antibodies used for immune reconstitution study

Panel for Immune Reconstitution study

	FITC	PE	PerCP	APC
Tube 1	IgG1	IgG1	IgG1	IgG1
Tube 2	na	CD19	CD3	na
Tube 3	CD4	CD8	CD3	CD45RO
Tube 4	CD4	CD8	CD3	CD45RA
Tube 5	na	CD16	CD3	CD56
Tube 6	Lineage	CD123	HLADR	CD11c
Tube 7	CD4	CD25	CD3	na

Data acquisition and analysis

The samples were acquired on FACS Calibur (Becton Dickenson, Mansfield, MA, USA). A total of 20,000 events were acquired for all the experiments and for rare populations 75,000 events were acquired. The data was analyzed using CellQuestPro software (Becton Dickenson, Mansfield, MA, USA).

Instrument setup and controls

- The CaliBRITE Beads were used to ensure that the instrument provides consistent readings from day to day by adjusting the gain settings and fluorescence compensation values to standard samples of known light scatter and fluorescent intensity.
- Proper compensation set up was done with single-stained compensation controls to correct the spill over which is necessary for proper antigen density measurements.

- Isotypic controls were also used to measure the level of non-specific background signal caused by primary antibodies.

3.8.1. Gating strategy and phenotypic analysis of lymphocyte populations

In order to identify lymphocyte populations, cells were first gated based on forward (FSC) and side scatter (SSC) properties on a dot plot. Lymphocyte subset percentages were calculated from the lymphocyte gate as appropriate (Figure 22) and absolute lymphocyte subset counts were calculated for the analysis.

Absolute count = TWBC x % positive of CD marker x % lymphocytes / 10000

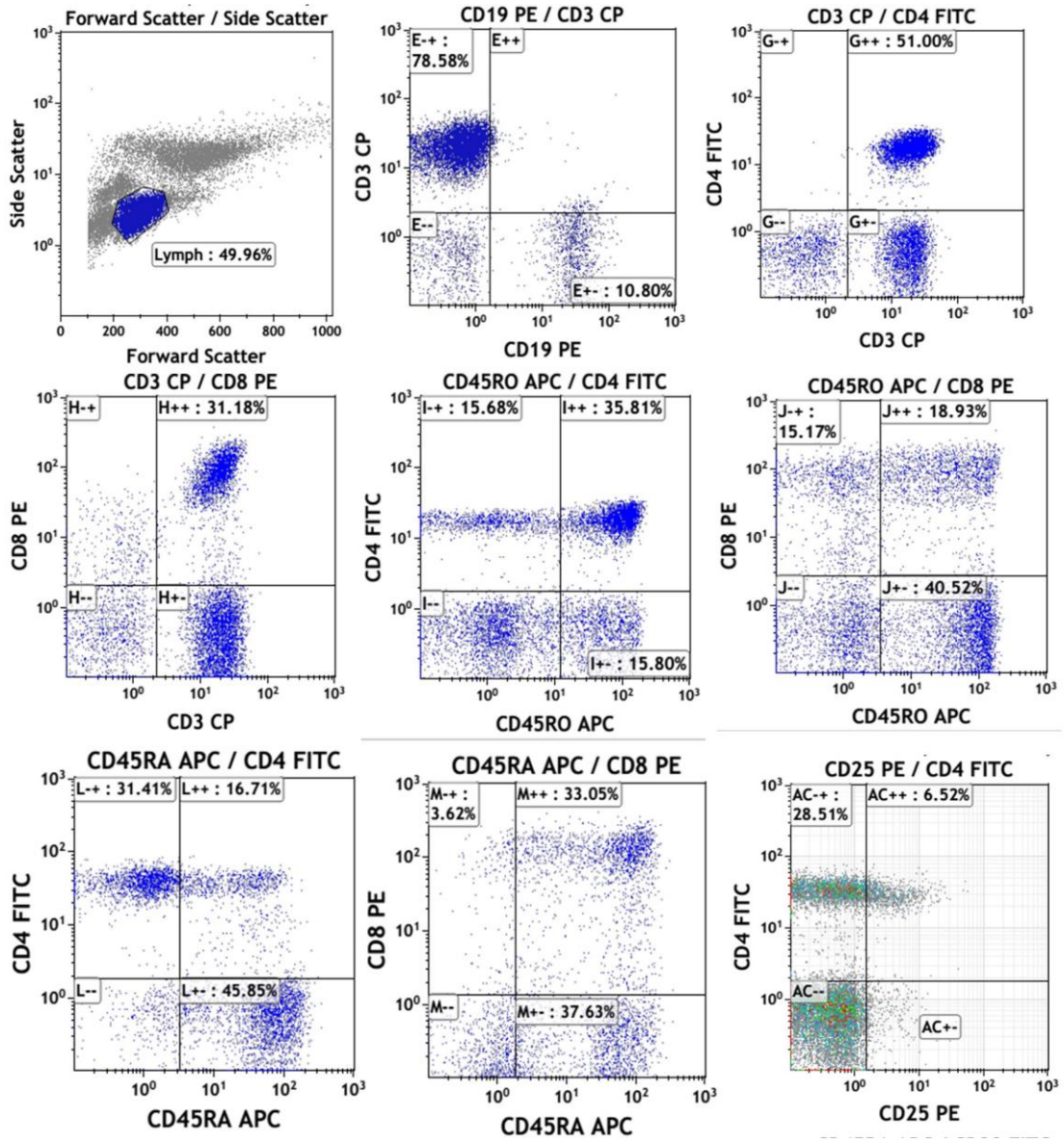


Figure 22. Representative dot plots of T cell and B cell immune subsets analyzed for immune reconstitution study*. Lymphocytes were gated based on forward (FSC) and side scatter (SSC) properties on a dot plot. Lymphocyte subset percentages were calculated subsequently.*

3.8.2. Enumeration of dendritic cells

Two different subsets of DC (monocytoid $Lin^{-}HLADR^{+}CD11c^{+}$ and plasmacytoid $Lin^{-}HLADR^{+}CD123^{+}$) were evaluated by using the gating strategy as described in Figure 23**.** A side scatter/forward scatter plot including all leukocytes were gated excluding debris and the cells negative for lineage markers (Lin 1 antibody: CD3, CD14, CD16, CD19, CD20, CD56) were selected in anti-HLADR/Lin 1 plot and then analysed for $HLADR^{+}CD11c^{+}$ (monocytoid) and $HLADR^{+}CD123^{+}$(plasmacytoid) cell populations. Since DC occurs at a very low frequency in peripheral blood, a total of 75,000 events were acquired for the analysis. The white blood cell count (WBC) and the DC % of PB were used to calculate the absolute DC counts.

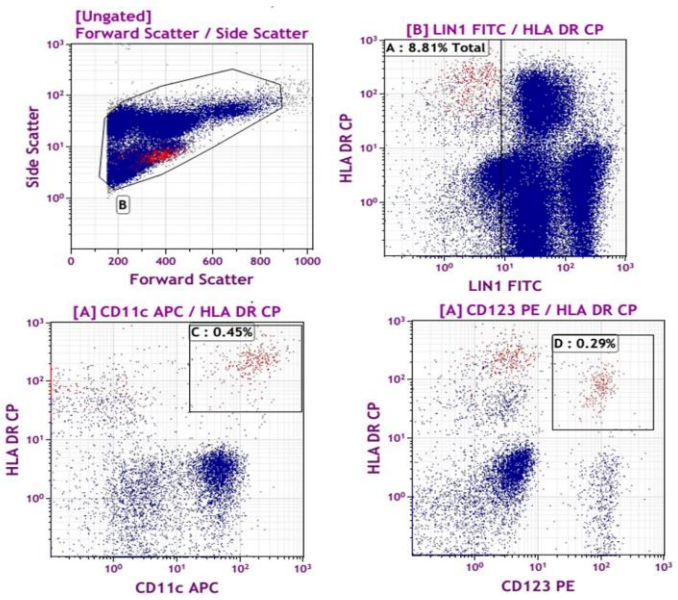


Figure 23. Gating strategy for enumeration of monocytoid ($HLADR^{+}CD11c^{+}$) and plasmacytoid ($HLADR^{+}CD123^{+}$) dendritic cells.

3.8.3. Phenotypic analysis of NK cell subsets

Human natural killer (NK) cells can be subdivided into different populations based on the relative expression of the surface markers CD16 and CD56. The CD56 dim NK cells represent at least 90% of all peripheral blood NK cells and are therefore the major circulating subset and the rest 10% are CD56 bright NK cells. In human peripheral blood five NK cell subpopulations can easily be distinguished by flow cytometry as follows (Zimmer et al., 2005).

(1) $CD56^{bright}$ $CD16^{-}$ (50-70% of $CD56^{bright}$)

(2) $CD56^{bright}$ $CD16^{+}$ (30-50% of $CD56^{bright}$)

(3) $CD56^{dim}$ $CD16^{-}$

(4) $CD56^{dim}$ $CD16^{+}$

(5) $CD56^{-}CD16^{+}$

$CD56^{+}CD3^{-}$ cells were gated from FSC/SSC lymphocyte gate and then were subsequently looked for CD56 and CD16 subsets as illustrated in Figure 24 and absolute counts were calculated. $CD56^{+}CD3^{+}$ NKT cells were also analyzed.

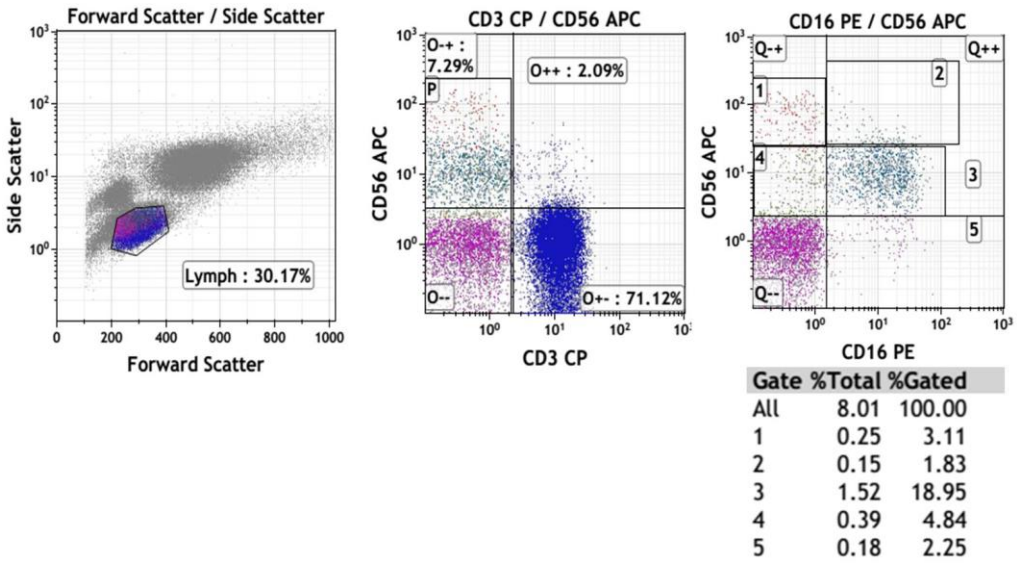


Figure 24. Gating strategy for NK cell subsets. *Lymphocytes are gated on FSC/SSC scatter and $CD56^{+}CD3^{-}$ populations are identified. $CD3^{-}$ lymphocytes are gated on another plot and NK cell subpopulations are determined in CD56 /CD16 plot. Also the $CD3^{+}$ T cells which express NK marker termed as NKT cells were determined from lymphocyte gate.*

3.8.4. Gating strategy for Myeloid-derived suppressor cells

MDSC subset was defined as previously reported having a phenotype of $CD14^{-}$ $HLADR^{-}CD33^{+}CD11b^{+}$ (Gabrilovich and Nagaraj, 2009). For MDSC gating, a side scatter/forward scatter plot including all leukocytes were gated excluding debris and the cells negative for CD14 and HLADR low are plotted and from that gate $CD11b^{+}CD33^{+}$ are selected as MDSCs. Figure 25 describes the gating strategy for MDSCs.

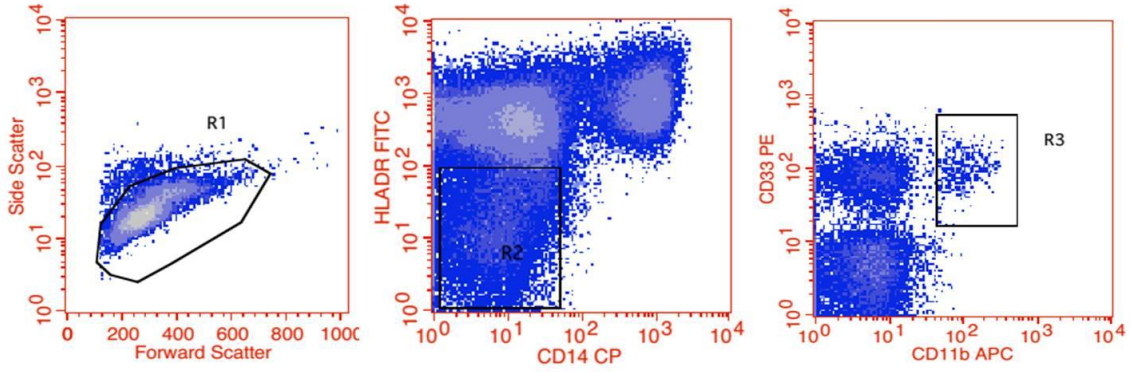


Figure 25. Representative dot plot defining the gating strategy for myeloid-derived suppressor cells (MDSC).

3.9. Preparation of proteins: human RAR alpha full length fused to GST and GST

GST-tagged proteins were constructed by inserting a gene or gene fragment into the multiple cloning site of one of the pGEX vectors. The vectors provide all three translational reading frames beginning with the EcoRI restriction site. The pGEX vectors were designed for inducible, high-level intracellular expression of genes or gene fragments. Expression in E.coli yields tagged proteins with the GST moiety at the amino terminus and the protein of interest at the carboxyl terminus.

The plasmids PGEX-2T-GST (5kb) and PGEX-2T full-length RARα with a concentration of 25ng/µl were being sent to us on Whatman #1 filter paper from Pr. Christine Chomienne lab which we are collaborating on this study. The plasmid was transformed with BL-21(DE3) competent E.coli and plated in Luria Broth (LB) agar

with ampicillin (50µg/ml). After the incubation for 16 hrs at 37°C single colonies were picked up followed by a quadrant streak again and incubated overnight at 37^0C. Single colonies were picked again and put in 5ml LB with ampicillin and incubated overnight at 37^0C at 225rpm. The OD should be 0.4-0.6. Glycerol stocks were made and stored at -80°C.

3.9.1. Large-scale protein preparation

Bacteria cells transfected with plasmid DNA were grown in 200ml LB overnight. After induction with IPTG (1mM) or without induction (4hrs at 37^0C/ 16hrs at 18^0C) the culture was spun down and the pellet was kept at -80°C.

Extraction of protein

The protein was extracted using B-PER Bacterial Protein Extraction Reagent (Thermo Fisher Scientific, Massachusetts, United States). It is a nonionic detergent-based solution that effectively disrupts cells and solubilizes native or recombinant proteins without denaturation. 300µl of B-PER reagent was added to the pellet (1.5ml culture) , mixed well and kept at RT for 5 min. The mixture was spun down at 13000rpm for 10 minutes, and the supernatant (containing the soluble fraction S) was frozen. B-PER reagent and lysozyme (6µl from 10mg/ml) was added to the pellet and incubated for 10 minutes. After centrifugation at 13000rpm for 10 minutes, the supernatant which contained the desired GST or the GST-RARα recombinant protein was then purified

using the GST spin purification kit (Thermo Fisher Scientific, Massachusetts, United States) and was stored at -80^{o}C.

SDS-PAGE Gel Electrophoresis

SDS-PAGE Gel Electrophoresis was used to separate the extracted proteins using a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins**.** The proteins were separated according to their molecular weight, based on their differential rates of migration through the gel under the influence of an applied electrical field. The protein samples were mixed with lamelli buffer and boiled in water bath for 10 minutes to denature them before loading them on the gel. The proteins were separated on a 7.5% SDS-PAGE. After running the gel, it is stained with Coomassie brilliant blue R-250 (Thermo Fisher Scientific, Massachusetts, United States) overnight and then destained to visualize the protein bands.

3.9.2. Western blotting

The purified human RARα and GST recombinant proteins were subjected to immunoblotting to confirm its presence by using anti-GST and anti-RARα antibodies. Protein lysates were quantitated using the Bradford assay for protein estimation. 50μg of total protein was used for western blot analysis. NB4 homogenates obtained by cell lysis in RIPA buffer (Sigma-Aldrich, SL, USA) with protease inhibitors (Roche, Basel, Switzerland) was used as control. After 20 min on ice, samples were centrifuged at 13,000 rpm for 10 minutes and supernatants were collected. The protein samples were resolved on 7.5% SDS-PAGE. The separated proteins were then transferred to

polyvinylidene difluoride (PVDF) membrane in transfer buffer. Following transfer, the membranes were blocked with non-fat milk (5%, 2 hrs at room temperature), followed by incubation with primary antibodies RARα C-20 (Santa Cruz, Dallas, USA) and GST B-14 (Santa Cruz, Dallas, USA) for overnight at 4^{o}C. The blots were then washed with TBS-T and probed with either anti-mouse or anti-rabbit or anti-human secondary antibodies conjugated with horseradish peroxidase (Cell Signalling Technology Inc., Massachusetts, USA) for one hour at room temperature followed by washing in TBS-T thrice (5 minutes each). The protein bands were detected by standard chemiluminescence method using SuperSignal West Femto Chemiluminescent (Thermo Pierce Femto, Rockford, IL, USA) and the images were captured using Fluor ChemQ system (Alpha Innotech, USA) provided with Alpha View® Q Software.

3.9.3. Detection of anti-RARα antibodies by ELISA

For anti- RARα antibody detection ELISA test was performed in 96-well plates coated with either GST or GST-RARα (obtained by fusion of a GST tag to the full-length RAR α, a kind gift from Pr. Christine Chomienne, France) (preparation of the protein mentioned above). For each serum, specific absorbance was calculated as the difference between duplicates of mean absorbance between GST-RARα and GST. This procedure was standardized in collaboration with the French Group Dr.Marie Helene Schlageter, Hospital Saint Louis, Paris. The steps involved in protein coating and antibody detection were as follows:

1. **Coating step**: 500ng/well of RARα protein or 1000ng/well of GST protein diluted in 0.05mM Bicarbonate buffer (100µl/well) was added and incubated overnight at 4^{o}C.

2. **Washing step**: The plate was blot dried next day and the wells were washed with 250µl/well washing solution four times.

3. **Blocking step**: The plate was saturated with 5% BSA solution (200µl/well) and kept for blocking for 2 hours at 37^{o}C.

4. **Primary Antibody step:** The plate was washed, and monoclonal antibody MAb 9alpha (1/5000 dilution) as positive control or human /mice serum (1/100 dilution) to be tested was added (100µl/well) and incubated at 37^{o}C for 1 hour.

5. **Secondary antibody step**: Secondary antibody coupled with HRP (1/10000 dilution) was added (100µl/well) and incubated at 37°C for 1hour.

6. **Detection:** The wells were washed again and fresh TMB solution was added (200µl/well) and incubated for 20minutes at room temperature. 50µl/well of 1M H_2SO_4 was then added to stop the reaction and the plate was read at 450nm vs 550nm in Spectramax M4 multimode reader (Molecular Devices, CA, USA). Specific absorbance (SA) was calculated as the difference between duplicates of mean absorbance with and without GST-RARα.

The specific absorbance was calculated as follows: $SA = A_{RAR\alpha} - A_{GST}$. All the tests were performed in duplicates and normal serum samples were also tested.

3.10. In vitro cytotoxicity assay

To determine the *in vitro* sensitivity of the malignant cell lines towards ATO used in this study, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was done (Biotium, Inc. CA, USA). It is a calorimetric assay for assessing cell viability and cytotoxicity of drugs. The yellow tetrazolium MTT is reduced by metabolically active cells by the action of dehydrogenase enzymes to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometrically. The intensity of the color developed is directly proportional to the number of viable cells.

To determine the inhibition concentration 50 (IC50), the concentration of ATO at which the viability of cells are reduced to 50% when compared to untreated control cells. The cell lines used in this study (NB4, UF1, NB4EVAsR1, K562, U937, HL60, Jurkat E6.1, SUP-B15 and NK92MI), were treated with increasing concentrations of ATO. The ATO concentration range, seeding density and the incubation time were arrived after series of standardization experiments performed in the laboratory. Briefly, $1x10^5$ cells /well were seeded in flat-bottomed 96-well tissue culture plates in the presence of increasing concentrations of ATO (Intas Pharmaceuticals, India) ranging from 0.12–6.0 µM. Cells without drugs were included as controls and culture medium alone was used as blank. The plates were incubated for 48 hours with drugs at 37^{o}C in humidified air containing 5% CO2, after which 10 µl of MTT reagent was added and the plates were incubated for 4 hours. Only viable cells can reduce MTT tetrazolium salt to

purple/blue formazan crystals. The formazan crystals were dissolved using 100µl of acidified Sodium dodecyl sulfate (0.01 N HCl-10% SDS) by incubating it for a minimum of 2 hours. The optical density was measured spectrophotometrically at 550nm with reference at 620nm using Spectramax M4 multimode reader (Molecular Devices, CA, USA). The analysis was done by non-linear regression curve fit and the graph and the IC-50 values were generated using Graph Pad Prism software (Version 6, La Jolla, CA, USA).

3.11. NK92MI cell line characterization

NK92MI cell line was used for all the functional assays and are characterized for its surface markers and receptors using the monoclonal antibodies CD2, CD7, CD11a, CD28, CD45, CD56, Nkp44, Nkp46, Nkp30, NKG2D, KIR2DL1, KIR3DL1/DL2, KIR2DS4, CD94 and CD226 ,CD1, CD3, CD4, CD5, CD8, CD10, CD14, CD16, CD19, CD20, CD34, HLA-DR and KIR2DL2 conjugated with FITC, PE, PerCP or APC (details of the antibodies and panel in table 6)

3.12. NK cell functional assays

3.12.1. NK cell cytotoxicity assay

To study the NK cell cytotoxic effector functions, flow cytometric based cytotoxic assay which measures target cell lysis induced by NK cell cytotoxic activity was performed using the ACT 1 assay for cytotoxicity kit (Cell Technology, Mountain View, CA, USA). A cell tracking dye CFSE is utilized to label the target cell population

(NB4) and thus separating them from the effector cell population (NK). Carboxyfluorescein diacetate succinimidyl ester (CFSE) passively diffuses into cells. It is colorless and nonfluorescent until the acetate groups are cleaved by intracellular esterases to yield highly fluorescent carboxyfluorescein succinimidyl ester. The succinimidyl ester group reacts with intracellular amines, forming fluorescent conjugates that are well retained and can be fixed with aldehyde fixatives.

After the experimental protocol, 7AAD (live/dead) was added to measure cell death. 7AAD only enters membrane compromised cells and binds to DNA. Flow cytometric analysis was done to gate on the target cells and measure 7AAD negative vs. 7AAD positive cells (Figure 26).

Target cell labeling

1. To stain the target cells (leukemic cell lines) 1x 10^6 cells were resuspended with PBS and CFSE stain was added in 1:4 ratio (4µl of CFSE + 16µl of PBS)

2. The cells were incubated at 37° C for 15 minutes in the dark .

3. After the incubation, the cells were centrifuged and washed with PBS and resuspended in 10% RPMI media for 30 minutes.

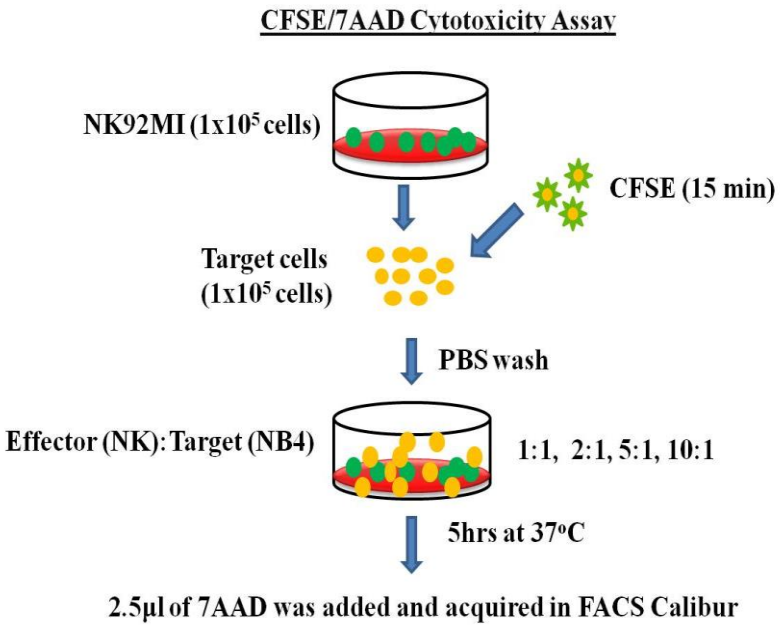


Figure 26. Assay protocol for CFSE/7AAD cytotoxicity assay. *The labeled target cells (1x 10^5) and effector cells (NK cell line) (1x 10^5) were cocultured in different ratios 1:1, 2:1, 5:1, 10:1 in the 24 well plate with 500µL 10% RPMI media. At the end of incubation at 37°C for 5 hours, the cells were washed, and 2.5 µl of 7AAD was added to the samples and acquired in FACS Calibur.*

NK cell cytotoxicity assay with myeloid and lymphoid cell lines

The cytotoxic activity of NK92MI (NK cell line) against six different myeloid (K562, U937, HL60, UF1, NB4 and NB4VMAsR1) and two lymphoid cell lines (Jurkat E6.1, SUP-B15) was assessed using the CFSE/7AAD cytotoxicity assay (assay protocol explained above). Briefly, NK cell line NK92MI (1x10^5) were seeded in 24-well plates in 500µl MEM supplemented with 10% FBS. Target cells (1x 10^5/500 µL/well in 10%RPMI) pre-treated with CFSE for 15 minutes at 37ºC and washed with PBS were then added to the effector NK cells at a ratio of 1:1, 2:1, 5:1and 10:1. The cells were

then co-incubated for five hours at 37^0C. After incubation cells were spun down and resuspended in PBS. After adding 2.5µl of 7AAD, the cytolytic activity of the NK cells was analyzed by flow cytometry using FACS Calibur (Becton Dickinson, San Jose, CA, USA).The percentage of cytotoxicity of the NK cell line is then calculated by the following equation.

The percentage of cytotoxicity of the NK cell line = 7AAD positive (UR quadrant)/ 7AAD positive (UR) + 7AAD negative (LR) X 100. The spontaneous death of the target cells is subtracted as background control. Figure 27 illustrates the gating strategy to assess the NK-mediated cytotoxicity.

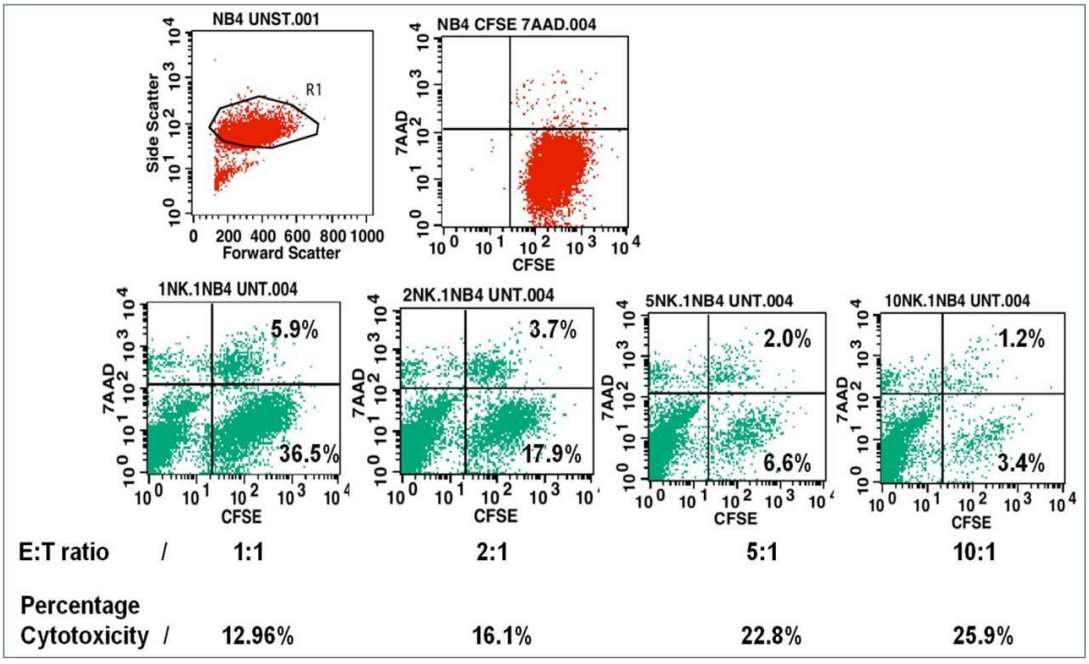


Figure 27: Illustration of gating strategy to assess NK cell mediated cytotoxicity using a CFSE/7AAD assay system *(NB4 cells stained with CFSE). Lower panel illustrates the effect of different NK cell (E) to NB4 cell (T) ratios.*

In a parallel set of experiments, the myeloid and lymphoid leukemic cell lines were pre-treated with 1μM ATO overnight for 12 hours and cytotoxicity will be measured as described above. Similarly, in another set of experiments, NK cell line was treated with 1μM ATO overnight for 12 hours, and cytolytic activity towards the leukemic cell lines were measured.

3.12.2. NK cell proliferation assay

NK cell line NK92MI ($1x10^6$) were left untreated or treated with 1μM ATO and seeded in 24-well plates in 500μl MEM supplemented with 10% FBS and checked for the proliferation. Briefly, the cells were stained with CFSE as mentioned in the above protocol and the intensity of CFSE stain was measured by flow cytometry using BD FACS Calibur at FL1 channel at 24, 48 and 72 hrs respectively. The reduction in the intensity of CFSE was plotted as histograms which is directly proportional to the cell proliferation.

3.12.3. NK cell degranulation Assay

A hallmark of NK cell activation is degranulation, the release of lytic granules (perforins and granzymes) to the surface of target cells. The inner surface of the granule is coated with CD107a (lysosome-associated membrane protein 1or LAMP-1), a highly glycosylated protein of the lysosomes. After degranulation, CD107a is exposed on the surface of the cytotoxic lymphocyte where it might protect the outer membrane from perforin-mediated damage. Externalization of CD107a has proven to be a marker of

degranulation of NK cells. This enables the direct detection of NK cytotoxicity which correlates with those of the standard cytotoxicity assay (Alter et al., 2004).

Degranulation of NK cells was assessed by flow cytometry-based assay. NK cell line was plated in 96-well U-bottom plates at 5×10^5 cells/well in the presence of fluorescein isothiocyanate (FITC)-labeled monoclonal antibody (MAb) against CD107a (BD Pharmingen, San Diego, CA) and was resuspended in 200µl media. Degranulation was induced by adding target cells (NB4, NB4EVAsR1,UF1, K562, U937, HL60 and Jurkat E6.1) (5×10^5 per well, effector/target [E:T] ratio=1:1). The contents of each well were mixed once with a pipette, and the plates were then centrifuged at 200*g* for 1 min, followed by incubation for five hours in a CO_2 incubator (5% CO_2, 37°C). The cells were then incubated with CD56 PE for 20 minutes followed by PBS wash and were acquired and analyzed in FACS Calibur. The percentage of $CD107a^+$ NK cells among all NK cells was measured. In another set of experiments the target cells are treated with 1µM ATO for 12 hours and then proceed for degranulation assay as explained above.

3.12.4. Genotyping of human KIR genes

The presence or absence of 15 human KIR genes plus two pseudo genes were analyzed by sequence-specific primers (SSP-PCR) using KIR typing kit (Miltenyi Biotech, Bergisch Gladbach, Germany) (Table 5). Briefly, the Enzyme Mix was lyophilized in the wells of the 96-well KIR Typing PCR plate. To set up the KIR typing PCR reaction, the Resuspension Buffer was mixed with the template (100ng/µl genomic DNA) and dispensed into the wells of the PCR plate (Figure 28). The Enzyme Mix

contains Taq DNA-Polymerase and loading buffer. Thus, the PCR products can be directly subjected to electrophoretic analysis in agarose gels (2%).

Table 5. List of the KIR genes and controls and its respective lanes in the KIR typing plate and the amplicon size.

Gene name	Lane	Amplicon size (bp)
2DL1	1	148
2DL2	2	145
2DL3	3	161
2DL4	4	221
2DL5	5	136
2DL5A	6	~ 1820
2DL5B	7	~1805
2DS1	8	148
2DS2	9	177
2DS3	10	172
2DS4del	11	204
2DS4ins	12	209
2DS5	13	179
3DL1	14	131
3DL2	15	232
3DL3	16	204
3DS1	17	149
2DP1	18	240
3DP1	19	237
Genomic DNA control for contamination	20	260
Positive control (β-actin)	21	400
Negative control	22	-

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	1	9	17	1	9	17	1	9	17
B												
C			19									
D			20									
E			21									
F			22			22			22			22
G												
H	8	16		8	16		8	16		8	16	

Figure 28. The format of the KIR typing plate*. Four patient DNA samples can be analyzed in each plate. 25 µL Resuspension Buffer-DNA mix was dispensed into wells 1-21. Well no 22 served as the negative control.*

The thermal cycler profile constitutes an initial denaturation of 95ºC for 60 seconds followed by 28 cycles of denaturation at 94ºC for 20 seconds, annealing at 63ºC for 20 seconds and extension at 72ºC for 90 seconds.

Interpretation of gel electrophoresis results

Every KIR typing PCR reaction contains an internal control, generated by a second pair of PCR primers. Thereby, two PCR fragments should be generated from the sample template: One is derived from the KIR specific primers and the second is amplified by the internal control primers. Positive gene-specific amplifications were identified by the presence of PCR gene-specific amplicons of the correct size, whereas the absence of gene-specific amplicons implied the absence of the genes identified in a

given primer mix. Figure 29 shows the representative image of PCR products ran in 2% AGE.

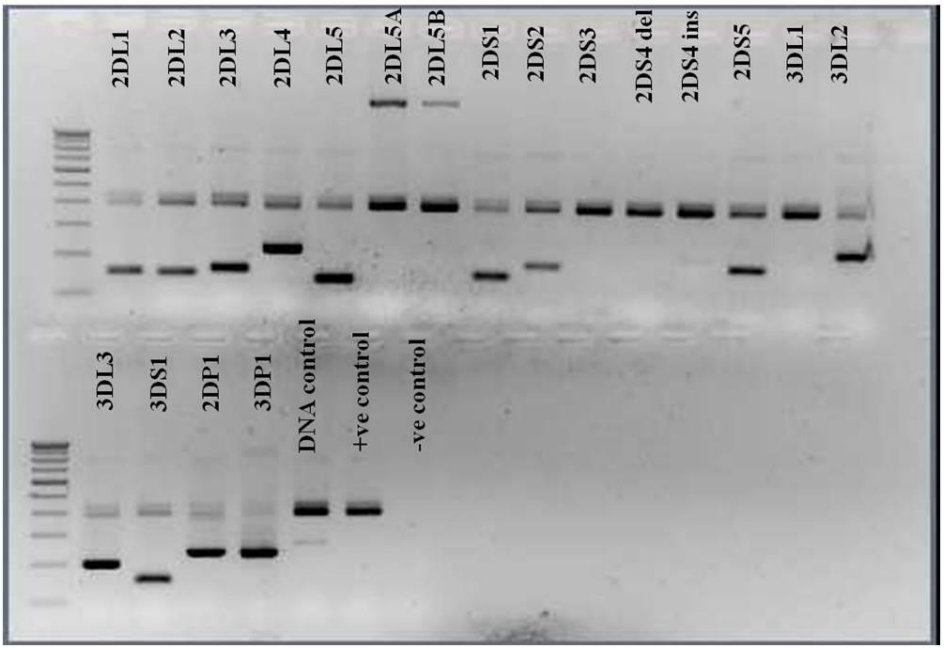


Figure 29. Representative image of PCR products of KIR Typing ran in 2% AGE. *1st lane shows the molecular weight marker.*

KIR haplotypes

Based on the KIR gene content, genotypes are broadly classified into Haplotype A and Haplotype B. The basis of each A or B haplotype consists of four framework genes: *KIR2DL4*, *KIR3DL2*, *KIR3DL3* and *KIR3DP1*. The A haplotype is non-variable in its gene organization, using up to eight genes: those of the framework and *KIR2DL1*, *KIR2DL3*, *KIR2DS4* and *KIR3DL1*. The B haplotype is defined by the presence of one or more of the genes encoding activating KIRs, *KIR2DS1/2/3/5*,

KIR3DS1 and the genes encoding inhibitory KIRs, *KIR2DL5A/B* and *KIR2DL2*. The pseudogenes KIR2DP1and KIR3DP1 were present in all patients. B haplotypes have more genes encoding activating KIR than A haplotypes.

3.12.5. NK cell receptor profiling on NK92MI cell line

To check for the expression of activating and inhibitory receptors on NK 92MI cell line, the cells were stained with antibodies to activating receptors NKp30 (CD337), NKp44 (CD336), NKp46 (CD335), NKG2D (CD314) and DNAM 1 (CD226) and inhibitory receptors, KIR2DL1 (CD158a), KIR2DL2 (CD158b), KIR2DS4 (CD158i), KIR3DL1/DL2 (CD158e/k) and NKG2A (CD94) (Table 6) with the respective isotypic control antibodies followed by incubation in dark for 20 minutes. Unbound antibodies were removed by washing with PBS and data acquisition and flow cytometric analysis were carried out in BD FACS Calibur using BD CellQuest Pro software. The cell line was also treated with 1μM and 2μM of ATO for 6 hours and 24 hours and then checked for the receptor expression as explained above.

Table 6. Panel for NK receptor and ligand immunophenotyping.

Panel for NK cell receptors and ligands

	FITC	PE	PerCP	APC
Tube 1	IgG1	IgG1	IgG1	IgG1
Tube 2	CD226	CD314	CD3	CD56
Tube 3	na	CD335	CD3	CD56
Tube 4	na	CD336	CD3	CD56
Tube 5	na	CD337	CD3	CD56
Tube 6	CD158b	CD158a	CD3	CD56
Tube 7	na	CD158e/k	CD3	CD56
Tube 8	HLA-ABC	CD158i	CD3	CD56
Tube 9	CD94	CD112	CD3	CD56
Tube 10	na	MIC a/b	CD3	CD56

3.12.6. NK ligands expression on malignant cell lines

$1x10^6$ cells were seeded in a 24 well plate and were left untreated or treated with 1μM and 2μM ATO for 6 hours and 24 hours. The cells were then washed and stained with antibodies to CD112 (Nectin 2-ligand for DNAM-1), MICA/B (ligand for activating NK cell receptor NKG2D) and HLA Class 1 or the respective isotypic control antibodies followed by incubation in the dark for 20 minutes. Unbound antibodies were removed by washing with PBS and data acquisition and flow cytometric analysis were carried out in BD FACS Calibur using BD CellQuest Pro software (Figure 30).

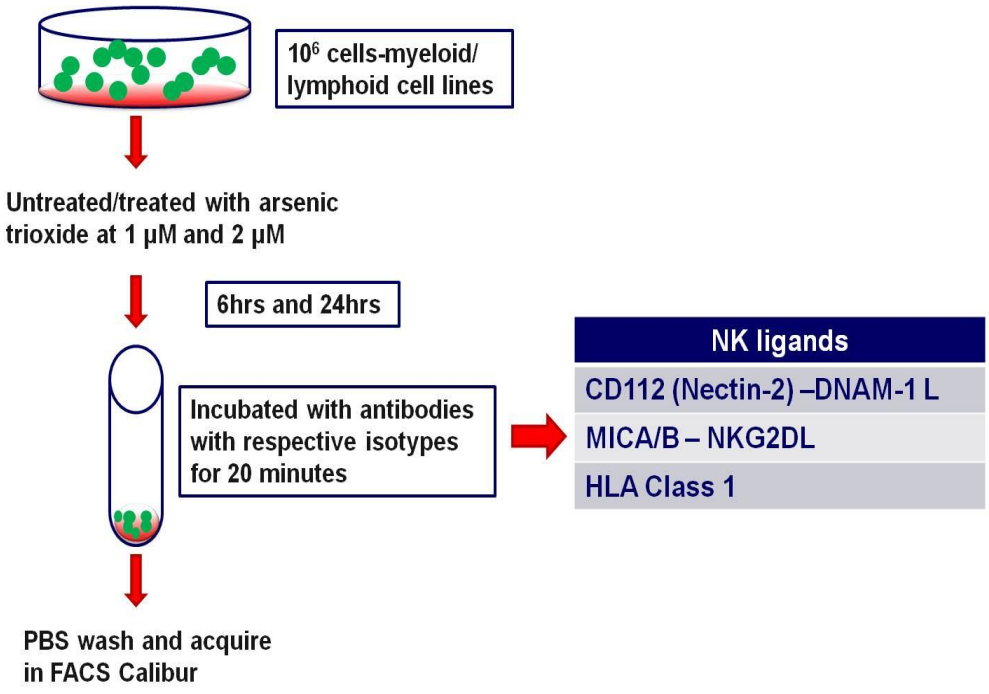


Figure 30. Schematic representation of NK ligand profiling in leukemic cell lines.

3.13. Stem cell-derived NK cell differentiation and characterization

For NK cell isolation and expansion, umbilical cord blood samples were collected from the Department of Obstetrics and Gynaecology, Christian Medical College, Vellore after informed consent.

1. 20 ml of cord blood sample was transferred to a sterile falcon tube and was passed through 70-micron filter.

2. Density gradient centrifugation was done by layering the sample on ficoll (3:1 ratio) and was spun at 400g for 30 minutes.

3. The buffy coat was separated and washed with PBS. The cells were again washed in 1x PBS+1mM EDTA+2%FBS (recommended medium) and proceeded for sorting. The final cell suspension has $1.8x10^7$ cells resuspended in 1ml media for CD34 positive selection (Easy Sep Human CD34 positive selection Kit, Stem cell Technologies, Vancouver, Canada).

4. 100µl of Easy Sep positive selection cocktail is added to 1ml cell suspension in Falcon polystyrene round bottom tubes and incubated at room temperature for 15 minutes.

5. 50µl of Easy Sep magnetic nanoparticles was then added and incubated for 10 minutes.

6. The cell suspension was made up to 2.5ml with the recommended medium and kept the tube in the Easy Sep magnet for 5 minutes.

7. Pour off the supernatant in one continuous motion by inverting the magnet. The magnetically labeled cells which remained in the tube were resuspended again and repeat the separation 2-3 times.

8. Finally, the positively selected CD34 cells were pelleted and resuspended in NK differentiation medium (10%RPMI+10ng SCF+30ng FLT3+50ng IL-15) and kept in an incubator at 37ºC with 5%CO_2.

9. The sorted cells were again subjected to a second step of sorting next day as explained in above steps from 4-7 and were resuspended again in the above-mentioned medium.

10. The sorted CD34 cells were checked for purity and expression of cell surface markers by flow cytometry.

10. The NK differentiation was assessed on Day 8, Day 14 and Day21.

11. The sorted CD34 cells were also exposed to 0.5uM ATO, and then NK differentiation was assessed.

The sorted CD34 positive cells were assessed for its purity and viability by flow cytometry using the markers CD45, CD34, CD3, CD19, CD56, CD33 and 7-AAD. The cells were also assessed for its NK differentiation on Day 8, day 14 and Day 21.

3.14. Quantitative real-time PCR (RQ-PCR) for NK cell transcription factors

Even though the precise hierarchy of the transcription factors that control NK cell maturation is not known, we evaluated the expression of some of the major transcription factors Eomes, Ikzf2, Prdm1, Ets1, Tox, Klf4 and Tbx21 involved in differentiation and maturation. The T-box transcription factors Eomesodermin (Eomes) and T-bet direct the fate and function of cytotoxic cell lineages including NK cells and CD8+T cells (Gordon et al., 2012). Furthermore, T-bet controls the developmental stability of immature NK cells, while Eomes regulates NK maturation. Klf4, Tox, Tbx21 and Ets1 are involved in the maturation of pre-NK to iNK (invariant NK) and Prdm1 and Ikzf2 are essential for NK cell function (Luevano et al., 2012).

RNA was extracted from the CD34 cell culture on day 0 and day 14 either untreated or were exposed to 0.5μM ATO and expression levels of NK transcription factors were determined based on TaqMan® Gene Expression Assays. Hs00172872_m1, Hs00212361_m1, Hs00153357_m1, Hs00358836_m1, Hs00428293_m1, Hs01055573_m1, Hs00203436_m1 were used for detecting Eomes, Ikzf2, Prdm1, Klf4, Ets1, Tox and Tbx21 mRNA expression respectively and was normalized to the house keeping gene GAPDH (Taqman assay ID Hs 02758991_g1). Gene expression was calculated by 2^ΔΔCT method where the difference in threshold cycle (ΔCT) values of the target gene and the housekeeping gene GAPDH for each sample was normalized to ΔCT value of the untreated sample (CD34 day0).

3.15. Animal experiments

All the experiments involving animals were performed according to the Institutional guidelines for animal care specified at Christian Medical College (Vellore, India) and the study design and euthanasia protocols were approved from Animal Ethics Committee IAEC (IAEC approval number: 2/2012). FVB/N mice strains were purchased from Charles River Laboratories International.Inc. USA. Experimental mice were housed in Laboratory Animal Facility (LAF), Center for Stem Cell Research (CSCR, Vellore, India) and maintained under pathogen-free conditions at 22-24°C in individually ventilated cages with free access to water and food. Mice at 6 to 8 weeks of age were used in all the experiments. Intraperitoneal, intravenous or intramuscular interventions were done during the experimental procedures as per the animal facility rules. Retro-orbital blood samples were collected for experiments. At the end of the studies, mice were euthanized by carbon dioxide (CO2) inhalation.

3.15.1. Establishment of APL transplantable mouse model

We took advantage of a mouse transplantable acute promyelocytic leukemia (APL) model that has proven to be a very robust and reproducible *in vivo* pre-clinical model of all the current and prospective therapeutic approaches of this leukemia as a proof-on-concept for other malignancies. Transgenic APL mice bearing the human PML-RARα cDNA (bcr1) with hMRP8 promoter were previously constructed in FVB/N inbred strain of mice, and a transplantable model was established in which 100% of the mice die of the disease (Brown et al., 1997). To allow reproducible APL development,

mouse APL blasts were obtained as a kind gift from Dr. Christine Chomienne and Dr. Rose-Ann Padua, Inserm UMR-S940 Institut Univérsitaire d'Hématologie, Hôpital Saint-Louis with the permission from Dr. Scott Kogan and Dr.Michael Bishop (University of California, San Francisco) and was sent by liquid nitrogen from the laboratory of France. As a part of the collaborative work with the French group, 6-8week old wild type FVB/N mice was injected intravenously in the tail with 5x 10^4 APL blasts without conditioning with either radiation or chemotherapy. After one week, leukemia was confirmed by the following parameters and thus re-established the APL mouse model at our center (Figure 31).

3.15.1.1. Confirmation of leukemia

a) Blood counts: White blood cell (WBC), hemoglobin, and platelet counts in peripheral blood (PB) were determined using an automated hematology analyzer (Horiba ABX ABC Vet Automated Veterinary Analyzer.)

b) Morphologic and histochemical analysis: BM was obtained by flushing long bones with RPMI media (L-Glutamine 200mM, 2% Fetal Bovine Serum, penicillin / streptomycin). The cells were washed with phosphate-buffered saline (PBS) at 400g for 10 min at 4°C. Peripheral blood smears or bone marrow cells cytospin were prepared and stained with Wright's Giemsa (Merck, New Jersey, USA). Intact spleen and liver were isolated and fixed in 10% buffered formalin and subsequently embedded in paraffin for hematoxylin and eosin (H&E) staining. Spleen size was compared with normal FVB/N and documented.

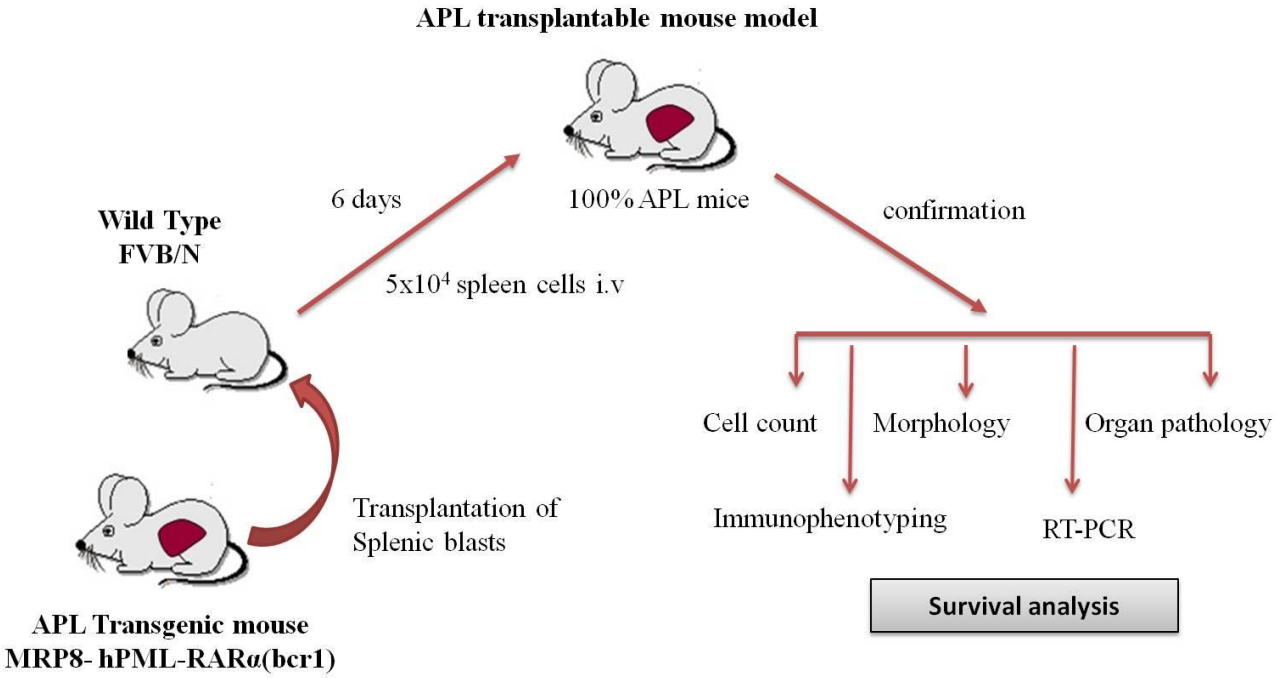


Figure 31. Establishment of a preclinical animal model of APL. *6-8week old wild type FVB/N mice was injected intravenously in the tail with 5x 10^4 APL blasts. After one week, leukemia was confirmed by different parameters.*

c) RT-PCR: RNA was isolated from PB and spleen samples and cDNA was prepared as explained previously. The PML-RARα fusion gene transcript bcr1were amplified by a two-step Qualitative nested reverse transcriptase as per the standard protocols in the lab. 10μl of PCR products were loaded on 2% agarose gel with ethidium bromide and run in 0.5x TBE buffer along with Step up ^{TM}100bp DNA ladder (Merck Millipore, NJ, USA) and visualized under UV light.

d. Immunophenotyping of immature CD117^{+} Gr1^{+} cells: Bone marrow cells were flushed from the murine long bones (tibias and femurs) or murine spleen cells and were harvested and disrupted in RPMI and filtered through nylon cell strainer (70μm) to obtain a single-cell suspension. The peripheral blood samples were collected from retro-orbital plexus and whole cell RBC lysis was done. After washing with PBS, PB cells/bone marrow mononucleated cells/ spleen cells ($1x10^{6}$cells) were incubated with Mac-1 (CD11b) antibody, c-Kit (CD117), CD45 and Gr-1 (myeloid differentiation antigen) antibodies (BioLegend, CA) conjugated with FITC, PE, PerCP and APC respectively for 20 minutes followed by RBC lysis and PBS wash. At least 10,000 events were analyzed from each sample on Gallios (Beckman coulter, CA, USA) and analyzed using Kaluza software (Beckman Coulter Life Sciences, Indianapolis IN).

3.15.1.2. Details of injections schedule

Substances: ATO, ATRA pellets, pVAX14 DNA plasmid vaccine, IL15

Doses: ATO- 5μg/g mice for 28 days continuously (diluted in PBS), ATRA-5mg 21day release pellets, DNA plasmid-100μg per mouse 3 doses with 20 day intervals. Recombinant mouse IL-15 (Biolegend, San Diego, CA) was administrated intra-peritoneally (*i.p.*) at the dose of 100ng/mouse (100μl) every 5 days for 5 doses.

Sites: ATRA - subcutaneous, ATO and IL15-intraperitoneal, DNA - intramuscular

Blood withdrawal volumes: 100-150μl

Sites: retro-orbital plexus

3.15.1.3. Survival analysis

The wild-type FVB/N after intravenous injection of $5x10^4$ APL blasts was monitored for survival.

Harvesting of cells and cryopreservation from APL mice

After the confirmation of leukemia, the APL mice were sacrificed and BM and spleen cells were harvested (as explained above) and cryopreserved (90%FBS + 10% DMSO) dimethyl sulfoxide) in -80^{o}C for future experiments.

3.15.2. Procedure for DNA vaccine preparation

Alkaline lysis method followed by cesium chloride (CsCl) based density gradient centrifugation was used for large scale preparation of pVAX14 DNA plasmids. Transformed E.coli cells were grown in 1L LB broth by overnight incubation at 37° C and continuous shaking at 220rpm. For plasmid isolation, bacterial cells were first lysed by treatment with lysozyme and subsequently by alkaline lysis (0.2N NaOH-1% SDS). Following cell lysis step, 3M sodium acetate and chloroform were added to separate the plasmid DNA from cell debris and denatured genomic DNA. Plasmid DNA was then precipitated by addition of 40% PEG. This solution was spun down and the pellet was dissolved in distilled water. The addition of 5.5M lithium chloride then facilitated the precipitation of RNA. Further isopropanol was added to the supernatant to separate out DNA, which was then dissolved in TE buffer (10mM Tris, 1mMEDTA, pH8). To this solution, about 10 grams of CsCl was added along with 10µl of ethidium bromide (EtBr). The tubes were sealed and subjected to centrifugation at 45,000rpm for nearly 15 hours in an ultracentrifuge using a 90Ti rotor (Beckman Coulter, Optima L-100K Ultra Centrifuge, USA). Under high centrifugal force, CsCl molecules dissociate and form a stable linear density gradient, with increasing density towards the bottom of the tube. DNA molecules placed in this gradient migrate to the point of equilibrium where they have the same density as the gradient (the neutral buoyancy or isopycnic point). The CsCl gradient can resolve DNA molecules with slight differences in density due to differing [G+C] content, or physical form (e.g., linear vs circular molecules).The DNA

band thus obtained was visualized in the presence of a UV lamp, which detects the fluorescence of intercalated EtBr. Later it was aspirated using a 21 gauge needle syringe and subjected to hydrated-butanol wash to remove EtBr contamination. DNA was further dialyzed in TE buffer overnight with continuous stirring, so as to remove butanol and residual salts. Finally, the purified supercoiled plasmid DNA was dissolved in TE buffer, and its concentration was determined using a spectrophotometer**.** The DNA plasmid was confirmed by restriction digestion and are stored in -20^{0}C in aliquots.

3.15.3. Immunophenotyping of T memory cells

10µl whole blood was directly stained with the respective antibodies and was incubated for 20 minutes followed by RBC lysis and PBS wash. The cells were acquired and analyzed in BC Gallios flow cytometer. For T memory cells, the lymphocytes were first gated on CD4/SSC and then the CD4 cells were gated for CD44^{high} CD62L^{dim} population.

3.15.4. Experimental protocols

3.15.4.1. Combination therapy with ATO, ATRA and DNA vaccine

Protocol 1

6-8 week old wild type FVB/N mice (n=34) was injected intravenously in the tail with 5x 10^4 blasts (thawed and resuspended in PBS)(Day 0) and are separated into five groups: a) placebo, b) ATO, c) ATRA and d) ATO and ATRA and e)ATO+ATRA+DNA vaccine. ATO (Intas Pharmaceuticals) was administered by daily

intraperitoneal injection at the concentration of 5 µg/g mice from day 7 post injection of malignant cells for 28 days. ATRA was administered on Day 7 by subcutaneous implantation of a 21-d release pellet containing 5 mg ATRA (Innovative Research of America). 100µg of DNA was injected intramuscularly into the quadriceps of mouse with 20-day intervals for three cycles starting on Day 8 post injection of malignant cells. Control mice were treated with intraperitoneal injections of PBS. Figure 32 shows the flow chart of time points of mice sampling.

Disease status was monitored clinically and biologically in mice as described above. Blood was collected by retro-orbital bleeding on day 20, day 35 and day60 and analyzed for blood counts, immune response measurement by analyzing T-memory cells and serum was collected to analyze antibodies to RARα at the last time point in all living mice and duration of survival in each arm was documented.

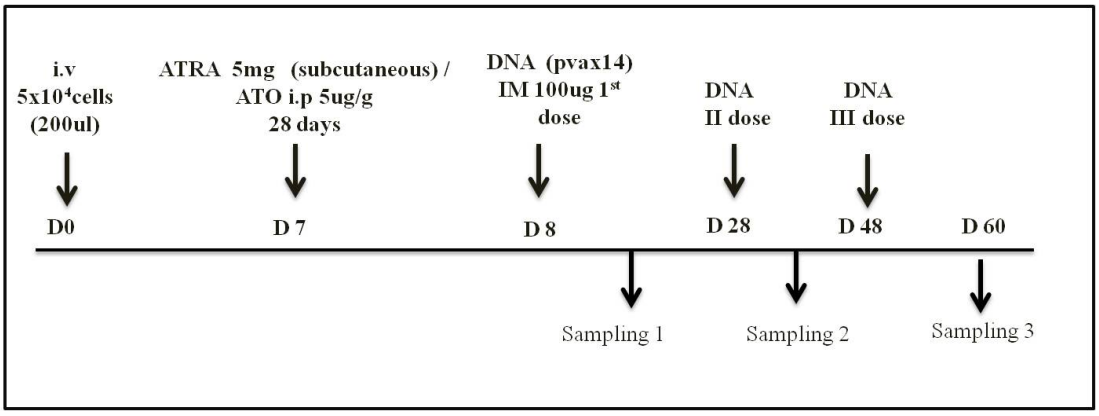


Figure 32 : Flow chart of leukemic FVB/N treated with ATO, ATRA and DNA vaccine. *The time points of administration of ATO, ATRA and DNA and the sampling time points are mentioned.*

Protocol 2

In the next set of experiments, 6-8 week old wild-type FVB/N mice injected with APL blasts were grouped as a) placebo (n=6), b) ATO (n=8), c) ATRA (n=5), d)ATO+ DNA vaccine (n=7) and e) ATRA+DNA vaccine (n=6). Interventions were done as described above and disease status was monitored clinically and biologically.

Challenge experiments on Long-term survivors (LTS)

Challenge experiments were done on LTS to assess the efficacy of clearance of leukemia initiating compartment by the treatment The LTS were challenged with 1 X 10^4 APL blast cells to the following groups:

a) ATO+ATRA (n=6) ATO+ATRA+DNA (n=2) ATRA+DNA (n=3) and ATRA (n=1) and placebo n=3) and looked for survival to explain the role of immune system in clearing up the leukemic cells. Number of mice developing leukemia and survival was documented in each group.

3.15.4.2. NK cell therapy in APL mouse model

Protocol 3

In another set of experiments, we have looked at the role of cellular therapy with NK cells along with the combination of chemotherapeutic agents. Briefly, 6-8 week old wild-type FVB/N mice (n=34) was injected intravenously in the tail with 5x 10^4 blasts and divided into following groups. a) Placebo, b) Placebo+NK cells, c) ATO, d) NK

cells+ATO, e) ATO+splenocytes. NK cells were isolated from the spleen of FVB/N as explained in figure 33. A total of 5x 10^5 NK cells were injected intraperitoneal for 3 doses with 10 days interval and sampling were done on day 15, day 27 and Day 45.

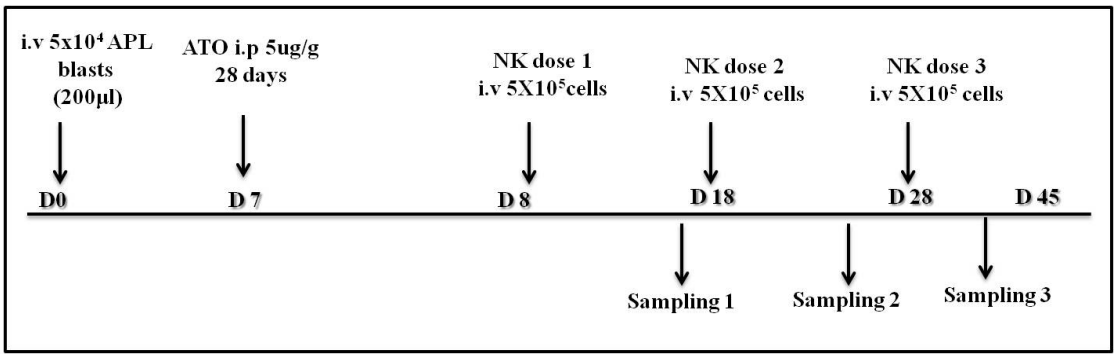


Figure 33: Flow chart of NK cell therapy in APL mouse model*. Schematic representation of time points of administration of three doses of NK cells with 10 day intervals.*

NK cell isolation from FVB/N spleen

Spleen was isolated from wild-type FVB/N and disrupted in 5ml of medium (PBS+2%FBS+1mM EDTA). The spleen cells were passed through a 70uM nylon mesh filter into a 50ml tube and removed the unwanted debris and clumps. The cells were then centrifuged and resuspended in medium. Optimal cell number for sorting should be 1 x 10^8 cells/ml and kept at room temperature (15-25^{o}C) and proceeded for the EasySep® Negative Selection Mouse NK Cell Enrichment Kit (Stem cell Technologies, Vancouver, Canada) for NK sorting.

Procedure for sorting

a) Single cell suspension was prepared at a concentration of $1x10^8$ cells/ml in the medium. Some cells were kept aside for staining for flow cytometry before sorting.

b) Cells were placed in a 5ml polystyrene falcon tube to be fit into the EasySep Magnet and 50µl/ml of Easy Sep Negative Selection Mouse NK Cell Enrichment cocktail was added and incubated at room temperature (15-25^{o}C) for 15 minutes.

d) After 15 minutes, EasySep Biotin Selection Cocktail at 200µl/ml was added and incubated for another 15 minutes at RT. Easy Sep D magnetic particles were also added at 200µl/ml and incubated for 10 minutes at RT.

f) The cell suspension was made up to a total volume of 2.5ml by adding the medium, and the tube was kept in the magnet for 5 minutes.

g) Invert the magnet and pour off the desired fraction into a fresh tube. A second round of magnetic separation was done and the enriched cells were then characterized by flow cytometry before use.

Assessing purity

The enriched NK cells (CD49b+CD3-) were assessed for purity by flow cytometry (BC Gallios, CA, USA) after staining with fluorochrome-conjugated antibodies against CD49b and CD3 and looked for percentage enrichment when compared with the splenocytes before sorting.

Protocol 4

NK cell therapy in APL mouse model with IL-15

To see the effect of IL-15 on NK cells, we injected the APL FVB/N with 100ng of recombinant mouse IL-15 intraperitoneal a total of 5 doses with 5 days interval along with ATO and NK cells (Figure 34). The groups were the following;

1. Leukemic FVB/n +ATO + IL-15 (n=6)

2. Leukemic FVB/n + ATO +IL-15+NK (n=6)

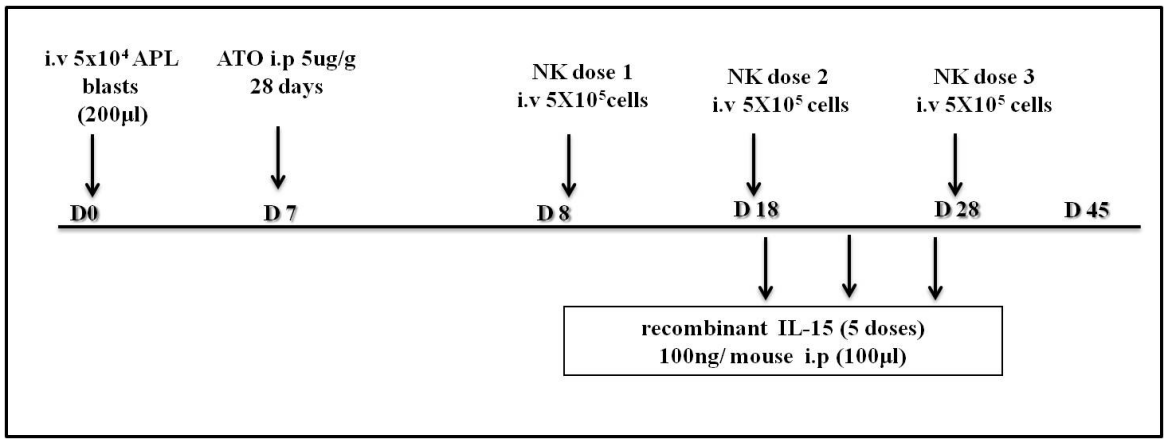


Figure 34. Flow chart of NK cell therapy in APL mouse model with IL-15. *Schematic representation of NK cell therapy in APL mouse model with three doses of NK cells and five doses of IL-15 in combination with ATO.*

3.16. Statistical analysis

Data was represented as mean of values ± SD (standard deviation) or as median values with range as indicated in the figure legends of the results section. Student's t-test or Mann-Whitney U test was used to statistically compare the continuous variables. For reconstitution graphs values were plotted as median with interquartile ranges. The relationships of clinical features to outcome were analyzed by Cox proportional hazard model. Logistic regression was used to compare the parameters with the end of induction RTPCR values. The probability of survival was estimated with the use of the product-limit method of Kaplan-Meier for overall survival (OS) and event free survival (EFS) and the significance was assessed by the log-rank test. All survival estimates are reported as ± 1SE. All P-values were 2-sided, with values of 0.05 or less indicating statistical significance. Statistical analysis used the SPSS 16.0 Software (Chicago, USA). Nonlinear regression curves and graphs were done with GraphPad Prism Software V6 (California, USA).

RESULTS

4.1. Patient demographics and characteristics

One hundred and twelve patients diagnosed with APL in our department from March 2010 to May 2015 were prospectively enrolled in the study after getting written and informed consent. Among them, 9 patients had an early death prior to the starting of treatment (less than 7 days) and 7 patients refused treatment at our center and were discharged against medical advice (DAMA). 96 newly diagnosed patients were included in the study. There were 47 males (48.9%) in the cohort with a median age of 32 years (range:2-59 years). Baseline clinical and laboratory parameters are summarized in Table 7.

All the 96 patients received ATO as a single agent as a part of the treatment as previously reported by us (Mathews et al., 2006). Among the 96 patients 6 died during induction therapy and 24 relapsed (25%). All the 24 had relapsed in the bone marrow, while 12 cases had a concurrent extramedullary relapse in the CNS. Of the 24 patients who relapsed 21 received salvage chemotherapy (one patient died before treatment and 2 were DAMA). 9 patients were consolidated with autologous stem cell transplantation and the rest were treated with combination of ATRA and ATO. The Kaplan-Meier estimate of event free survival and overall survival at 3 years was 66.7± 5.2% and 91.4± 2.9% respectively.

Table 7. Baseline clinical and laboratory parameters of newly diagnosed patients with APL. *The parameters mentioned are age, sex,hemoglobin (Hb), white blood cell count(WBC), platelet, lactate dehydrogenase (LDH), creatinine, partial thromboplastin time(PT), activated partial thromboplastin time (APTT), fibrinogen, % blasts in bone marrow and bcr1, bcr2 and bcr3 transcripts.*

PATIENT CHARACTERISTICS	
Parameters	**Value n (%) / median (range) (n=96)**
Age (years)	**32 (2-59)**
Sex: male	**47 (48.9%)**
Hb (g/L)	**79 (26-137)**
Total WBC ($x10^9$/Lt)	**10.6 (0.3-180)**
Platelet ($x10^9$/Lt)	**22 (3-77)**
LDH (U/L)	**651 (286-2143)**
Creatinine (mg%)	**0.9 (0.28-1.6)**
PT (secs)	**13.6 (9.3-26.7)**
APTT (secs)	**27.2 (23.1-38.3)**
Fibrinogen (mg/dl)	**163.8 (25-574)**
Blasts in BM (%)	**86 (48-100)**
RT PCR bcr1	**49 (51%)**
bcr2	**4 (4.1%)**
bcr3	**43 (44.7%)**

On a univariate analysis WBC count at diagnosis was significantly associated with relapse-free survival (RFS) where those patients who relapsed (n=24) had a higher median WBC count of 13.6 $x10^9$/L (range: 0.9-141.3 $x10^9$/L) when compared with non - relapse group (n=66) with a median WBC count of 8.45 $x10^9$/L (range: 0.3-180 $x10^9$/L) (p=0.055). Among the 82 cases available for evaluation at the time of completion of induction and documentation of hematological remission 46 (56.1%) were RT-PCR negative. Those patients who are RT-PCR positive at the end of induction (n=36) had a higher incidence of relapse when compared with RT-PCR negative patients (n=46) with a hazard ratio of 2.6 (95% CI 1.11- 6.16 p-value = 0.029). The median total WBC count is significantly lower in those patients who are RT-PCR positive at the end of induction when compared with the RT-PCR negative group [(7.1 $x10^9$/L (0.4-88.4) vs. 14.95 $x10^9$/L (0.3-141.3), p=0.047]. There was no correlation with other clinical parameters on the outcome. The effect of clinical and laboratory parameters on relapse-free survival is summarized in Table 8.

Table 8. Univariate analysis of clinical and laboratory parameters on relapse free survival as outcome. *p-value < 0.05 was considered significant.*

Variables	non-relapse n(%) / median(range) n=72	relapse n(%) / median(range) n=24	RFS HR(95%CI)	p value
Age (years)	32.5 (2-59)	29 (4-54)	0.9 (0.95-1.02)	0.321
Sex (male)	35 (48.6%)	12 (50%)	1.1(0.48-2.37)	0.872
Hb (g/L)	80 (26-137)	77 (40-128)	0.9 (0.78-1.13)	0.498
TC ($x10^9$/L)	8.95 (0.3-180)	13.6 (0.9-141.3)	1.0 (1.00-1.02)	0.055
Platelet ($x10^9$/L)	22 (3-68)	21.5 (4-77)	1.0 (0.98-1.04)	0.268
Blasts in BM (%)	87 (48-100)	83 (52-98)	0.9 (0.95-1.02)	0.569
LDH (U/L)	643.5 (286-2143)	705 (356-2060)	1.0 (1.00-1.00)	0.269
Creatinine (mg%)	0.9(0.46-1.6)	0.92 (0.28-1.4)	0.7 (0.13-4.16)	0.73
PT (secs)	13.5 (9.3-26.7)	14 (11.2-19.5)	1.0 (0.88-1.19)	0.733
APTT (secs)	27.45 (23.6-38.3)	26 (23.1-37.5)	0.9 (0.81-1.05)	0.217
Fibrinogen (mg/dl)	175.7 (25-574.1)	155.3(44-347)	0.9 (0.99-1.00)	0.302
RTPCR bcr1	36 (50%)	13 (54.2%)	1.0	
bcr2	3 (4%)	1 (4%)	1.0 (0.13-7.69)	0.996
bcr3	33 (45.8%)	10 (41.6%)	0.8 (0.37-1.93)	0.697
Days to HCR	45(38-60)	44 (37-55)	0.9 (0.81-1.01)	0.067
RTPCR end induction (positive)	21/66	15/24	2.6(1.10-6.15)	0.029

4.2. Evaluation of patterns of immune reconstitution in newly diagnosed APL patients treated with ATO based regimen

4.2.1 Differential reconstitution pattern of T cells, B cells and NK cells

Analysis of lymphocyte subset reconstitution patterns have shown that the absolute counts of all the subsets evaluated were below the normal range at diagnosis except for the dendritic cells, and NKT cells lie in the near normal ranges throughout the treatment. Following treatment, there was a differential pattern of immune reconstitution in different lymphocyte subsets (Figure 35a-c). Some of the prominent observations are summarized below.

Reconstitution pattern of NK cells revealed that NK cells ($CD56^+CD3^-$) were not recovered to normal ranges till the end of one-year post chemotherapy. Among the T cell subsets evaluated the $CD3^+CD4^+$ T helper cells recovered very slowly and almost in the lower limit of normal ranges even 6 months after completion of treatment (median 643.86 cells/µl, range: 203.36-1759.67 cells/µl vs. long term follow-up samples median 768.87 cells/µl, range: 275.72-2252.58 cells/µl) whereas the $CD3^+CD8^+$ T cytotoxic cells reconstitute before the start of consolidation therapy (approximately 3 months from diagnosis) (median 714.77 cells/µl, range:113.85-1930 vs. long term follow-up samples median 800.66 cells/µl range: 301.83-1753.86) (Figure 35a). The CD4/CD8 ratio remains inverted until the start of consolidation therapy. The CD4CD45RO and

CD8CD45RO memory T cells and CD4CD45RA, CD8CD45RA naive T cells had returned to levels within the normal ranges by pre-consolidation time point (Figure 35b). The absolute counts of B cells were low till remission and had recovered by starting of second maintenance. The median absolute lymphocyte count was below the normal ranges till remission and was recovered to normal levels by consolidation. The median CD4+CD25+ cells were recovered to normal levels by the end of induction (Figure 35c). The 25^{th} and 75^{th} percentile values of long term follow up patients were taken as the reference ranges for all subsets. The median absolute counts of each subset at different time points evaluated is described in detail in Table 9a and 9b.

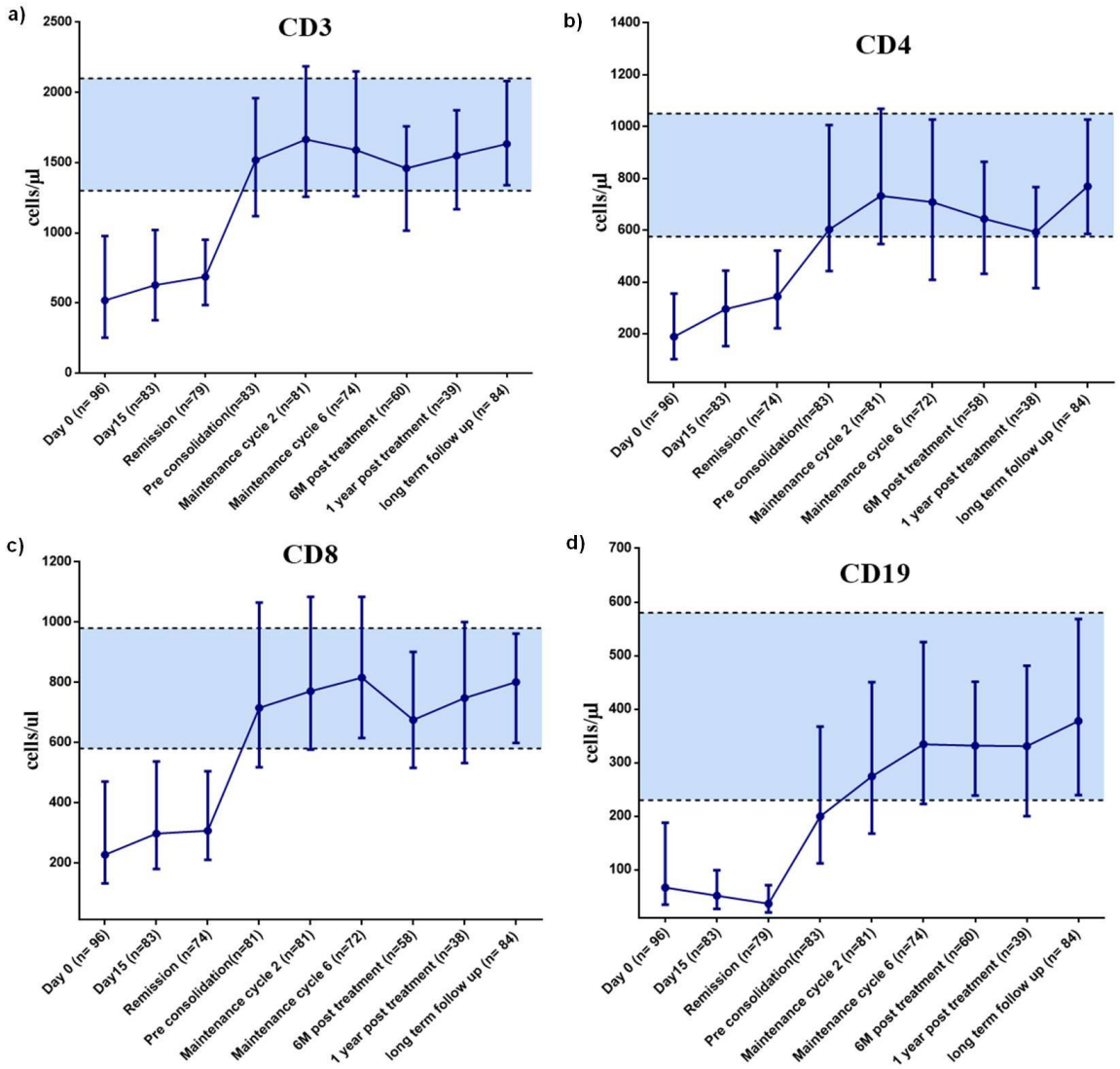


Figure 35a. The graphs showing the recovery of T cells and B cells at appropriate time points post treatment with ATO*. The error bars indicate median with interquartile ranges. The shaded region represents the 25th to 75th percentile values of long term*

follow-up samples serve as reference ranges. Number of samples available at each time points were mentioned in brackets. Absolute counts were given in cells per microlitre.

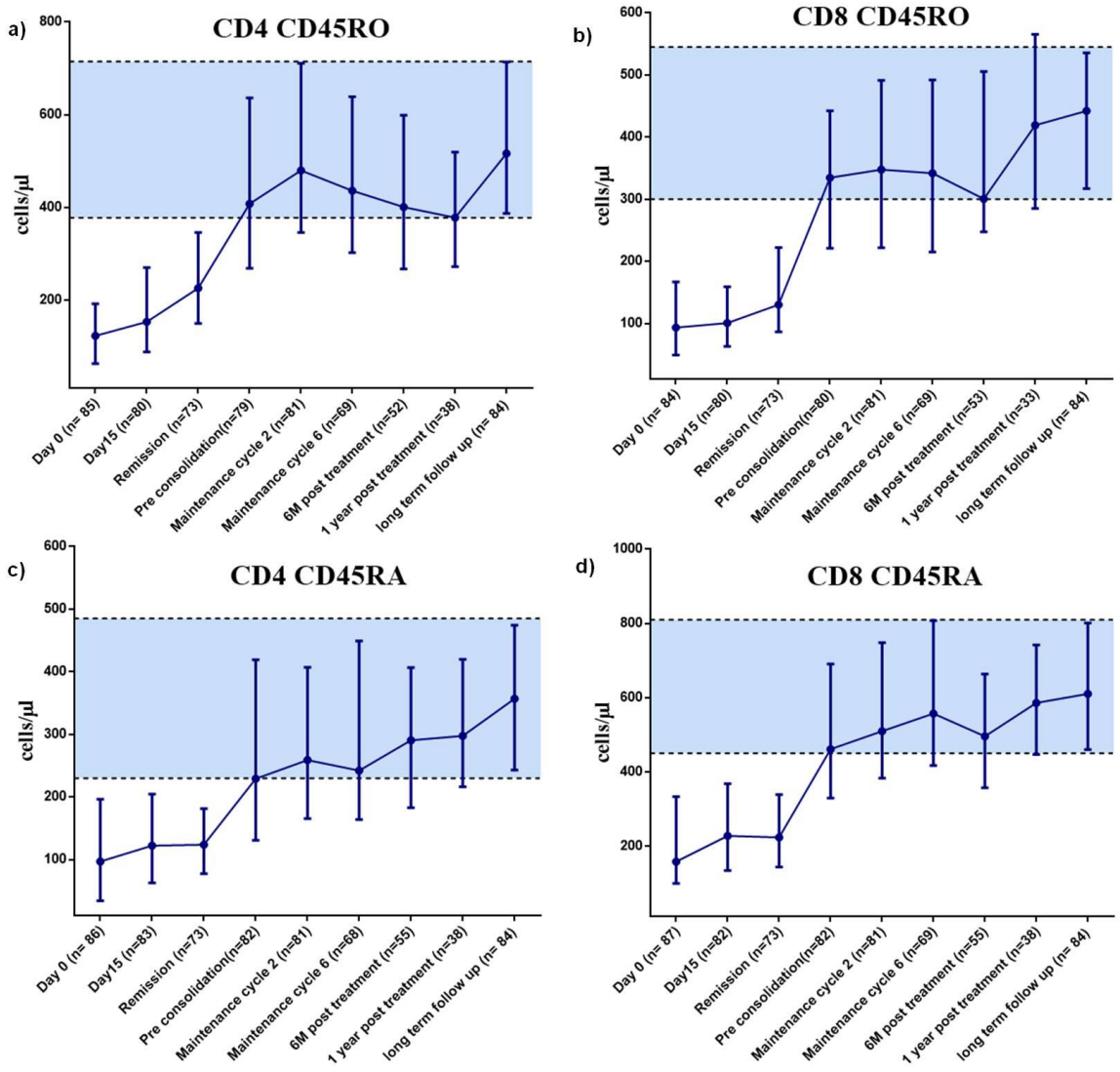


Figure 35b. The graphs showing the recovery of memory and naive T cells at appropriate time points post treatment with ATO*. The error bars indicate median with interquartile ranges. The shaded region represents the 25th to 75th percentile values of long term follow-up samples serve as reference ranges. Number of samples available at each time points were mentioned in brackets. Absolute counts are given in cells per microlitre.*

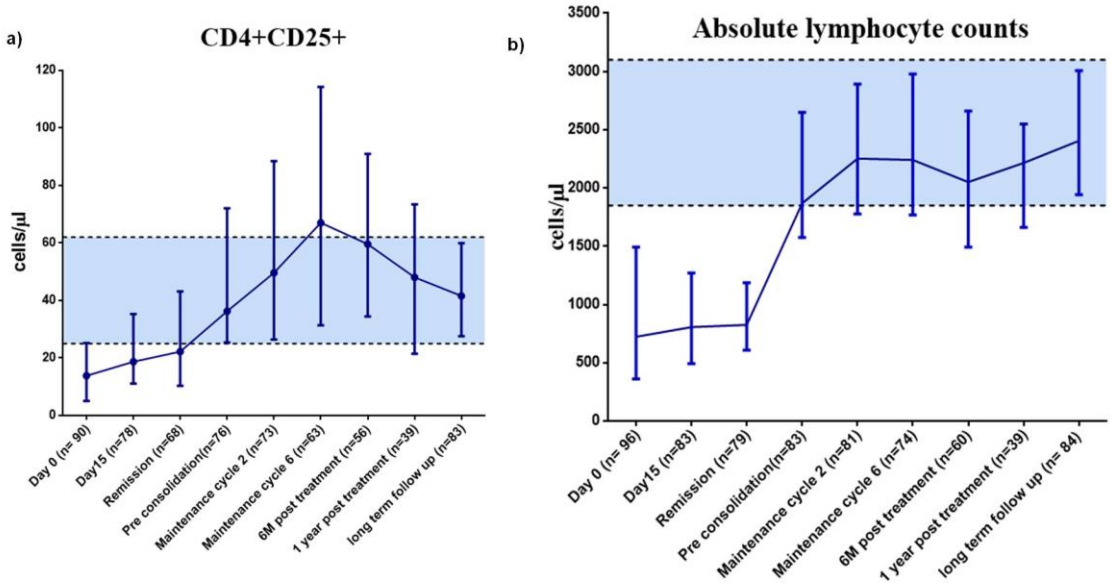


Figure 35c. The graphs showing the recovery of CD4+CD25+ activated T cells and absolute lymphocyte counts at appropriate time point post treatment with ATO*. The error bars indicate median with interquartile ranges. The shaded region represents the 25th to 75th percentile values of long term follow-up samples serve as reference ranges. Number of samples available at each time points were mentioned in brackets. Absolute counts are given in cells per microlitre*

4.2.2. Reconstitution pattern of NK cell subsets following ATO

NK cells are important effectors of innate immune system comprising 10–15% of all peripheral blood lymphocytes. By their phenotype and functional capacities, there are two major subsets, CD56 bright and CD56dim. The CD56 dim subset predominates (~90% of total NK cells) exhibiting a high cytotoxic potential and CD56 bright subset represents a precursor stage of terminally differentiated CD56 dim NK cells constituting less than 10% of NK cells and is immunoregulatory in function.

The median NK cell absolute counts in patients were in the below level of normal ranges even after completion of therapy (approximately a year) (median absolute counts at 6 months post treatment was 120.84 cells/µl, range: 27.85- 484.22 cells/µl vs. the long term follow-up samples median 177.85 cells/µl, range: 61.02-635.37 cells/µl). They recovered to normal levels only by one-year post therapy. We have also looked at the reconstitution pattern of two major subsets of NK cells, CD56bright and CD56dim subsets as shown in Figure 36a and 36b.

The absolute counts of the CD56 bright immature population ($CD56^{bri}CD16^{+}$, $CD56^{bri}$ $CD16^{-}$) was below the normal range until the end of induction and then they recovered to normal ranges before consolidation. The CD56 dim mature population ($CD56^{dim}$ $CD16^{+}$, $CD56^{dim}$ $CD16^{-}$) which contribute to the cytolytic activity and are differentiated from CD56 bright were below the normal ranges and had recovered only by the start of maintenance. The median absolute counts of $CD56^{dim}CD16^{+}$ cells were lower throughout the course of treatment when compared with the CD56bri subset. The $CD56^{+}CD3^{+}$ NKT subset lies almost in the normal limits till the end of treatment (Figure 4.2.1 and 4.2.2). The $CD56^{-}CD16^{+}$ subset which is a dysfunctional subset known for the impairment of NK cell function was in the normal ranges from consolidation till one-year post treatment (Figure 36c).

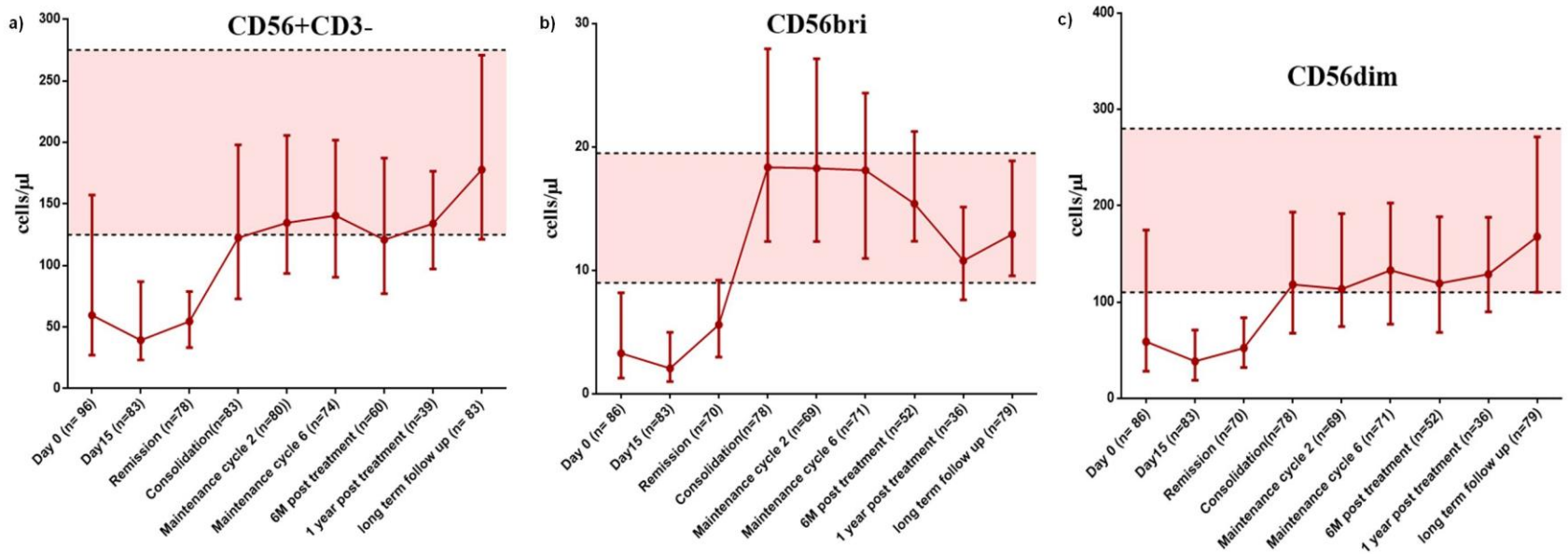


Figure 36a. The graphs showing the recovery of NK cells and subsets (CD56bright and CD56 dim) at appropriate time points post treatment with ATO*. The error bars indicate median with interquartile ranges. The shaded region represents the 25th to 75th percentile values of long term follow-up samples serve as reference ranges. Number of samples available at each time points were mentioned in brackets. Absolute counts are given in cells per microlitre.*

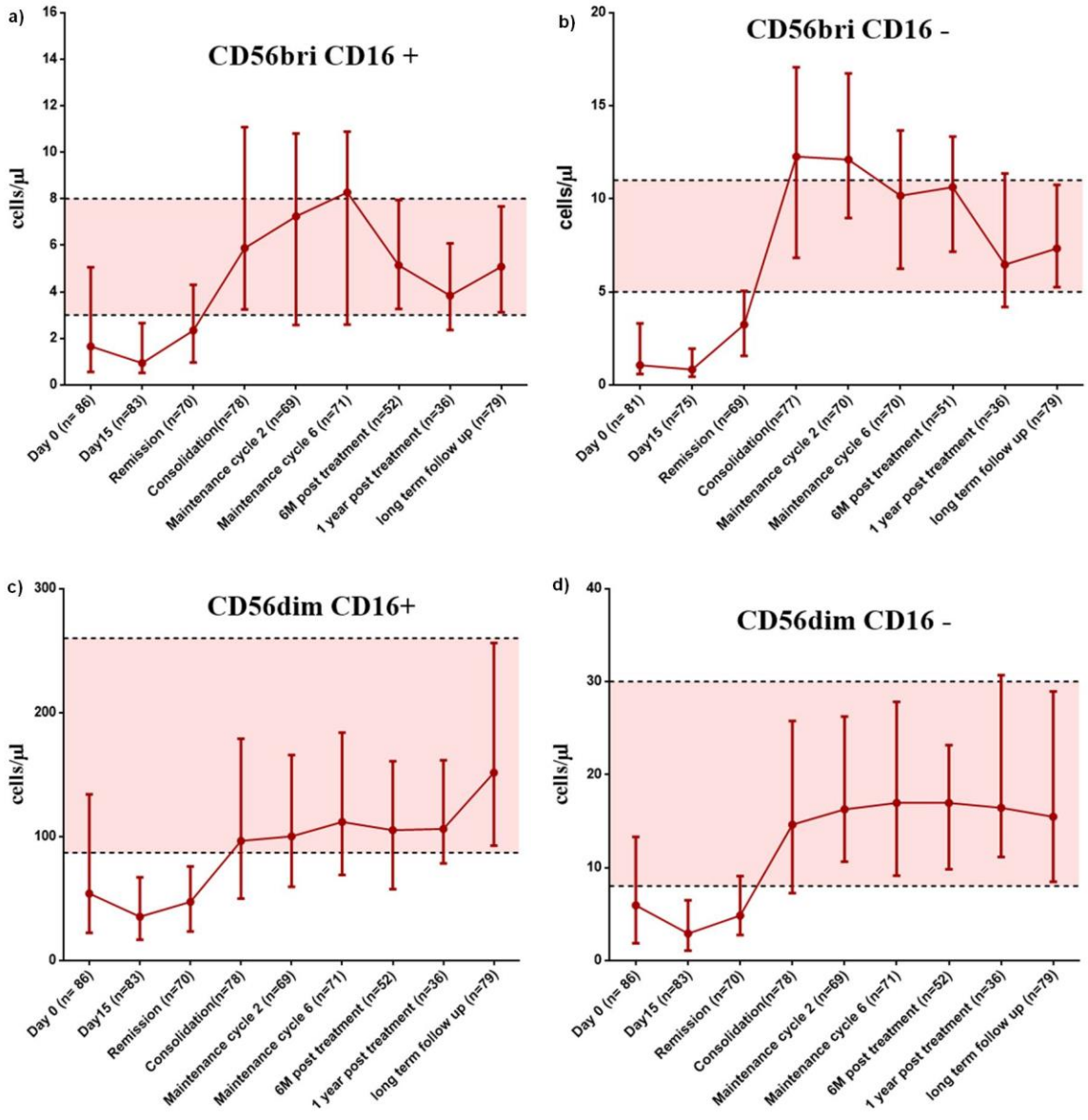


Figure 36b. The graphs showing the recovery of NK subsets (CD56bright and CD56 dim) at appropriate time points post treatment with ATO. *The error bars indicate median with interquartile ranges. The shaded region represents the 25th to 75th percentile values of long term follow-up samples serve as reference ranges. Number of samples available at each time points were mentioned in brackets. Absolute counts are given in cells per microlitre.*

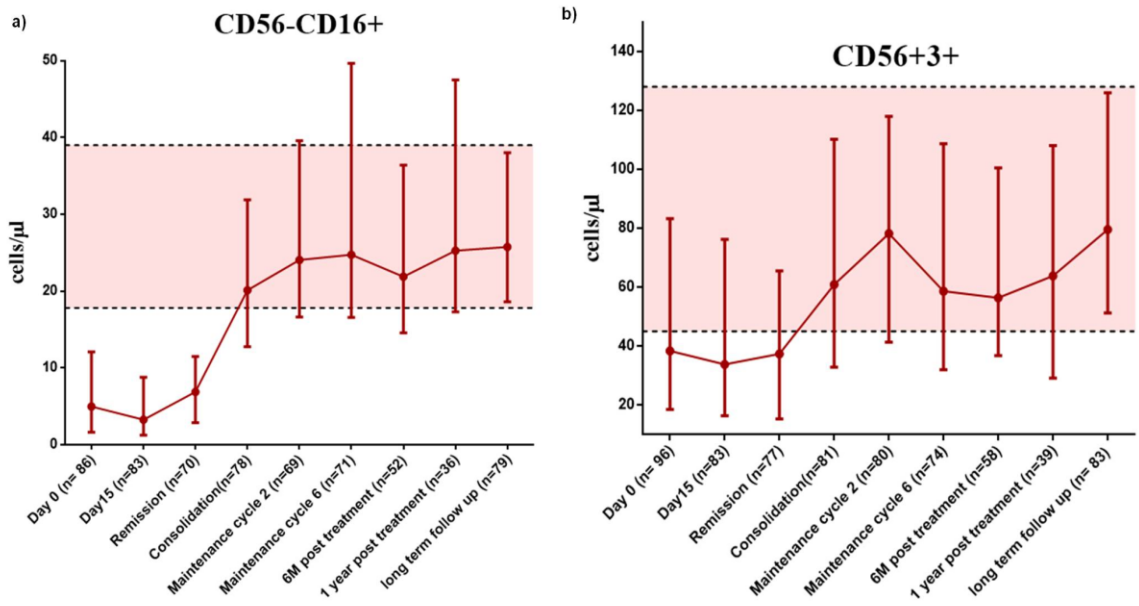


Figure 36c. The graphs showing the recovery of $CD56^{-}CD16^{+}$ and $CD56^{+}CD3^{+}$ NK subsets at appropriate time points post treatment with ATO. *The error bars indicate median with interquartile ranges. The shaded region represents the 25^{th} to 75^{th} percentile values of long term follow-up samples serve as reference ranges. Number of samples available at each time points were mentioned in brackets. Absolute counts are given in cells per microlitre.*

Table 9a. Table showing the median and range of absolute counts of immune subsets prior to diagnosis, on remission and pre-consolidation. *n denotes the number of patients at each time points for each subset.*

	Time points					
Immune subsets	Day 0 median(range)	(n)	remission median(range)	(n)	pre consolidation median(range)	(n)
CD3	518.33(27.05-6744.24)	96	686.82(150.26-4961.40)	79	1518.21(323.53-4725.44)	83
CD19	66.63(5.25-2528.69)	96	36.48(0.48-263.93)	79	199.61(10.79-1144.47)	83
$CD56^{+}CD3^{-}$	59.53(3.32-756.78)	96	54.6(6.63-383.73)	78	122.49(9.17-547.75)	83
$CD56briCD16^{-}$	1.06(0.06-49.66)	81	3.23(0.07-11.87)	69	12.26(0.49-66.42)	77
$CD56briCD16^{+}$	1.65(0.08-56.07)	82	2.34(0.09-21.44)	69	6.17(0.05-45.36)	76
CD56bri total	3.29(0.14-95.52)	82	5.59(0.26-26.14)	69	18.34(0.90-111.77)	77
$CD56dimCD16^{+}$	54.12(4.29-743.75)	86	47.40(2.79-369.36)	70	96.52(7.73-933.07)	77
$CD56dimCD16^{-}$	5.94(0.18-119.72)	85	4.84(0.37-49.47)	70	14.62(0.73-106.91)	77
$CD56^{-}CD16^{+}$	4.97(0.14-100.31)	86	6.88(0.63-72.08)	70	20.13(1.40-73.15)	77
CD56dim total	58.82(6.87-820.18)	86	52.27(4.72-380.76)	70	118.14(8.46-1039.98)	77
$CD56^{+}CD3^{+}$	38.35(1.06-374.68)	96	37.34(2.95-196.98)	77	60.85(13.98-606.50)	81
CD4CD45RO	123.59(10.34-1743.42)	85	226.2(48.59-765.73)	73	408.16(58.41-2553.88)	79
CD8CD45RO	93.56(6.44-1205.86)	84	130.40(34.99-466.69)	73	334.77(49.71-979.49)	80
CD4CD45RA	97.14(3.29-2328.29)	86	123.87(20.16-1809.55)	73	229.14(30.68-2155.54)	82
CD8CD45RA	158.71(10.84-2636.81)	87	223.65(24.94-1700.24)	73	461.29(102.03-1672.88)	82
CD4	188.68(8.61-3849.81)	96	344.0(78.57-2485.23)	74	602.57(89.83-3273.29)	83
CD8	226.91(12.65-4363.15)	96	307.04(21.59-1991.92)	74	714.77(113.85-1930)	81
CD4+CD25+	13.79(1.00-168.61)	90	22.22(1.46-87.72)	68	36.24(0.53-176.16)	76
CD11c+HLADR+Lin-	16.84(0.21-1962.8)	96	9.4(0.96-77.35)	75	27.72(2.99-161.50)	73
CD123+HLADR+Lin-	14.33(0.14-3815.1)	96	2.4(0.48-23.25)	75	3.39(0.75-22.41)	73

Table 9b. Table showing the median and range of absolute counts of immune subsets at 6 months post treatment, 1 year post treatment and long term follow-up samples. *n denotes the number of patients at each time points for each subset.*

	Time points					
Immune subsets	**6 months post treatment median (range)**	**(n)**	**1 year post treatment median (range)**	**(n)**	**Long term follow up (> 2 years) median (range)**	**(n)**
CD3	1459.24(562.60-3592.41)	60	1550.02 (734.03-2432.80)	39	1633.62(704.92-3462.71)	84
CD19	331.66(89.08-1053.86)	60	330.88 (87.85-755.87)	39	377.79(68.69-1819.71)	84
CD56+CD3-	120.84(27.85-484.22)	60	133.95 (43.60-307.53)	39	177.85(61.02-635.37)	83
CD56briCD16-	10.62(0.41-41.01)	51	6.87 (1.16-23.13)	36	7.33(1.85-47.77)	79
CD56briCD16+	5.14(0.34-28.95)	51	4.59 (0.44-12.64)	36	5.08(0.42-26.18)	79
CD56bri	15.41(1.23-48.95)	51	11.89 (1.75-31.71)	36	12.92(2.78-73.95)	79
CD56dimCD16+	105.21(10.59-438.44)	52	106.16 (34.23-328.54)	36	151.53(26.06-617.27)	79
CD56dimCD16-	16.96(3.02-78.33)	52	16.43 (4.70-103.35)	36	15.47(2.64-85.82)	79
CD56-CD16+	21.88(7.99-101.33)	52	25.27 (6.29-89.29)	36	25.75(5.57-104.59)	79
CD56dim	119.35(27.64-448.88)	52	128.96 (57.92-339.54)	36	167.83(44.57-623.14)	79
CD56+CD3+	56.35(13.09-365.15)	58	63.78 (10.63-234.02)	39	79.56(14.06-243.17)	83
CD4CD45RO	401.22(95.06-899.13)	52	378.20 (179.19-839.15)	33	516.52(155.49-1377.53)	84
CD8CD45RO	300.59(107-1303.47)	53	419.19 (157.31-763.23)	33	442.21(153.18-970.05)	84
CD4CD45RA	290.47(79.95-1573.98)	55	297.66 (126.4-659.81)	38	356.99(130.24-1077.92)	84
CD8CD45RA	496.13(164.71-1445.85)	55	585.75 (239.22-1084.56)	38	610.28(258.02-1090.49)	84
CD4	643.86(203.36-1759.67)	58	592.24 (268.79-1320.23)	38	768.87(275.72-2252.58)	84
CD8	674.84(222.29-1822.50)	58	747.73 (372.12-1630.29)	38	800.66(301.83-1753.86)	84
CD4+CD25+	59.58(5.87-231.01)	56	48.03 (12.17-374.48)	39	41.55(10.33-316.59)	83
CD11c+HLADR+Lin-	8.64(0.87-56.07)	53	20.9 (4.5-81.6)	39	23.25(6.7-93.6)	83
CD123+HLADR+Lin-	1.94(0.54-20.16)	51	4.92 (1.02-20)	39	5.82(1.34-18)	83

4.2.3. Reconstitution pattern of dendritic cell subsets

Monocytoid dendritic cells (MCDCs) and plasmacytoid dendritic cells (PCDCs) are potent APCs essential for primary cellular immune responses. Studies have shown increase or decrease in DC numbers in hematological malignancies, but the significance is not clearly known (Facchetti et al., 1989, Vuckovic et al., 1999). We observed that the absolute counts of MCDCs and PCDCs were in the normal ranges throughout the therapy. Before treatment the median absolute counts were higher and were reduced within 15 days of treatment and recovered to normal ranges by pre-consolidation (Figure 37).

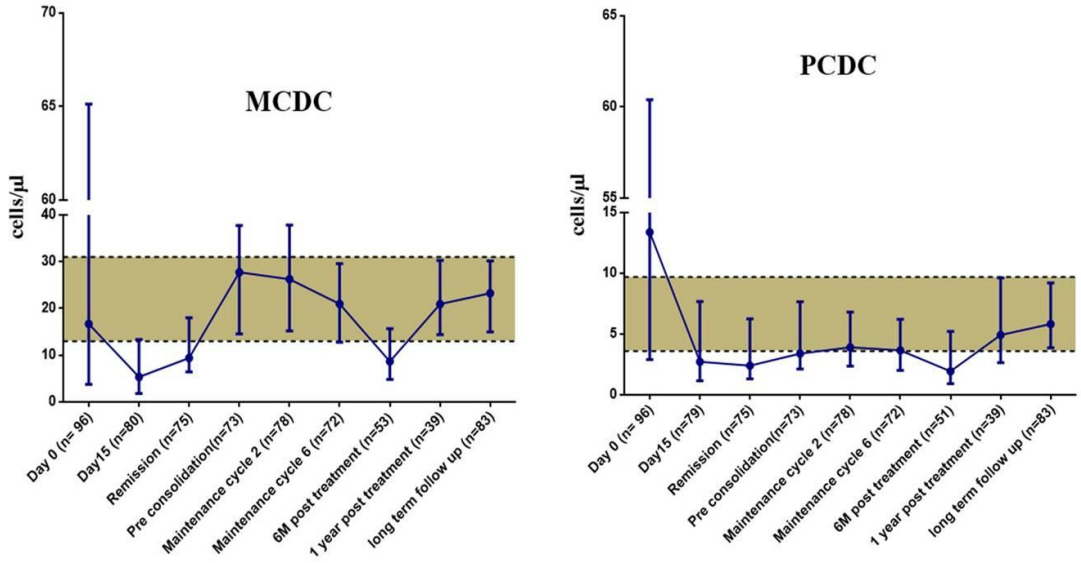


Figure 37. The graphs showing the recovery of dendritic cell subsets at appropriate time points post treatment with ATO*. The error bars indicate median with interquartile ranges. The shaded region represents the 25th to 75th percentile values of long term follow-up samples serve as reference ranges. Number of samples available at each time points were mentioned in brackets. Absolute counts are given in cells per microlitre.*

4.2.4. Impact of immune subsets on clinical outcomes

We investigated the impact of the immune subset reconstitution on relapse-free survival. The median absolute counts of $CD4^{+}CD25^{+}$ T cells at diagnosis was significantly higher in patients who relapsed (median 16.72 cells/µl, range: 3.37-130.75) when compared with the non-relapsed group (median 12.84 cells/µl, range: 1.0-168.6) (p=0.035). This observation was consistent even with the pre-consolidation absolute counts with a median of 68.62 cells/µl (range: 7.43-167.19) in relapsed patients when compared with non-relapsed group with median absolute counts of 35.51 cells/µl (range 0.53-176.16) (p=0.05). Similarly the median absolute counts of MCDC at diagnosis was significantly higher in relapse when compared to non-relapse group [median 36.9 cells/µl (range: 1.98-1962.8) vs 12.89 (range: 0.21-834.3) (p=0.001)]. At remission, the median absolute counts of CD4CD45RA naïve T cells were significantly lower in patients those who relapsed (median 114.8cells/µl, range: 40.6-1809.5) when compared with the non relapse group (median 124.2 cells/µl, range: 20.15-536.88) (p=0.038) (Figure 38).

We have also looked at the impact of NK maturation pattern on clinical outcomes. The $CD56^{-}CD16^{+}$ NK, a dysfunctional NK subset which impairs NK cell function is higher in relapse patients with median absolute counts of 35.03 cells/µl (range 9.65-114.71) when compare with non-relapse group (median 23.41cells/µl, range: 2.59-88.62) (p=0.013). Among the immune subsets evaluated at the time point of remission the absolute counts of CD56brightCD16- subset were significantly lower in

those patients who were RT-PCR positive at the end of induction with a median of 2.15 (0.06 - 11.87) cells/µl compared with those who were negative with a median of 3.92 (0.14 - 9.9) cells/µl (p=0.054). No other subsets evaluated at remission were significant.

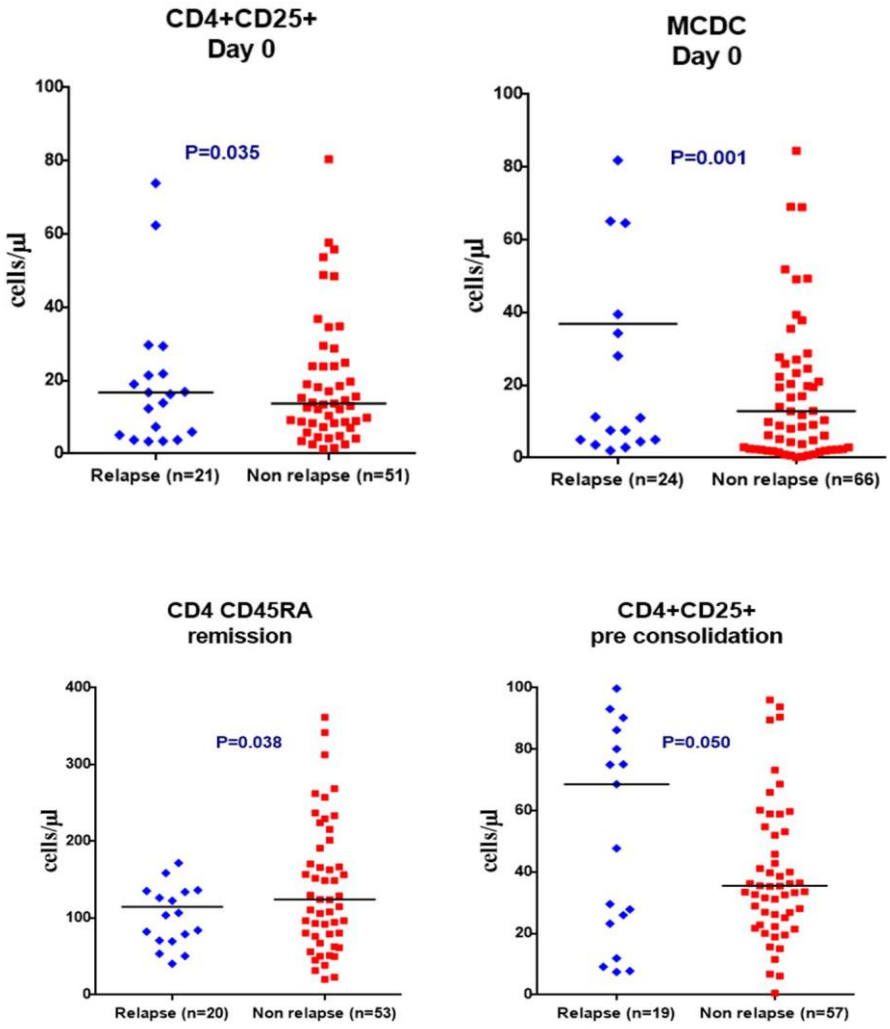


Figure 38. Graphs showing the impact of different immune subsets on relapse. *The median absolute counts of $CD4^{+}CD25^{+}$ T cells on day 0 and pre-consolidation are significantly higher in relapse group (p=0.035 and 0.050 respectively) and MCDCs on day 0 is significantly higher in relapse when compared to non-relapse group (p=0.001). At remission, CD4CD45RA subset is significantly lower in relapse cohort (p=0.038).*

4.3. Phenotypic heterogeneity of MDSC subsets

We evaluated peripheral blood (PB) samples from healthy controls (HC; n=10) and newly diagnosed leukemia patients (B-ALL=50, T-ALL=20, APL= 70 and AML=137) for the presence of MDSC (Table 10).

Table 10. Table showing the median MDSC percentage in peripheral blood of patients *with B ALL (n=50), T-ALL (n=20), APL (n=70) and AML (n=137) and in healthy subjects (n=10).*

Samples	MDSC (%) Median (range)
Healthy control (n=10)	0.7% (0.11-0.97)
B ALL (n=50)	0.14% (0.01-2.76)
T-ALL (n=20)	0.08% (0.01-1.1)
APL (n=70)	0.63% (0.04-5.94)
AML (n=137)	1.07 %(0.01-31.1%)

The median values were significantly lower in ALL and APL when compared with AML (P=0.001 and 0.005 respectively). There was no significant association among cases with ALL and APL with conventional risk factors. At phenotype level, there is significant heterogeneity between normal healthy controls (HC) and different leukemia's at diagnosis as illustrated in Figure 39. In APL, there was a very low level of this cellular subset at diagnosis.

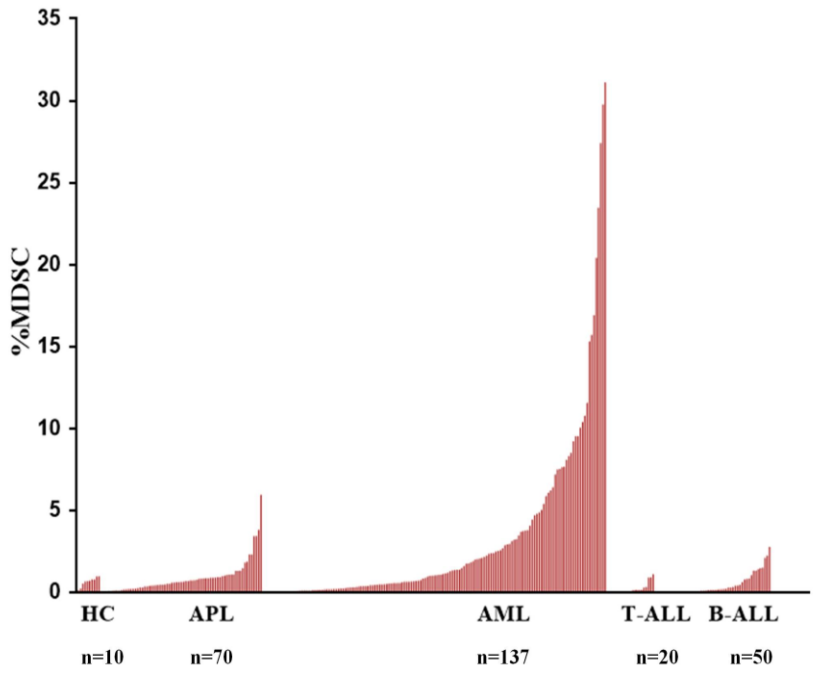


Figure 39: Graph showing the percentage MDSC in the peripheral blood in different subsets of leukemia *B ALL (n=50), T-ALL (n=20), APL (n=70) and AML (n=137) and in healthy subjects (n=10).*

Among newly diagnosed cases of AML, there is significant heterogeneity in the proportion of MDSC with 77 (56%) having values above the median of HC (Figure 40). There was weak, but significant positive correlation of MDSC with WBC counts at diagnosis and a significant negative correlation with the CD34 percentage at diagnosis (Figure 40). Comparison of AML cases in the highest quartile (MDSC > 3.74%; n=34) with the remaining patients (MDSC ≤ 3.74%; n=103) is summarized in Table 11. AML patients in the highest quartile of MDSC had significantly higher WBC count and a significantly lower CD34 count at diagnosis; there were also a significantly higher

proportion of cases that were NPM1 and FLT3 mutation positive in this quartile and similarly was the high-risk NPM1+and FLT3 mutation positive subset.

MDSC at diagnosis in PB samples were lower in ALL and APL. There was significant heterogeneity among cases with AML and a significant association with WBC count at diagnosis, NPM1 and FLT3 mutations and a significant negative correlation with the CD34 percentage at diagnosis.

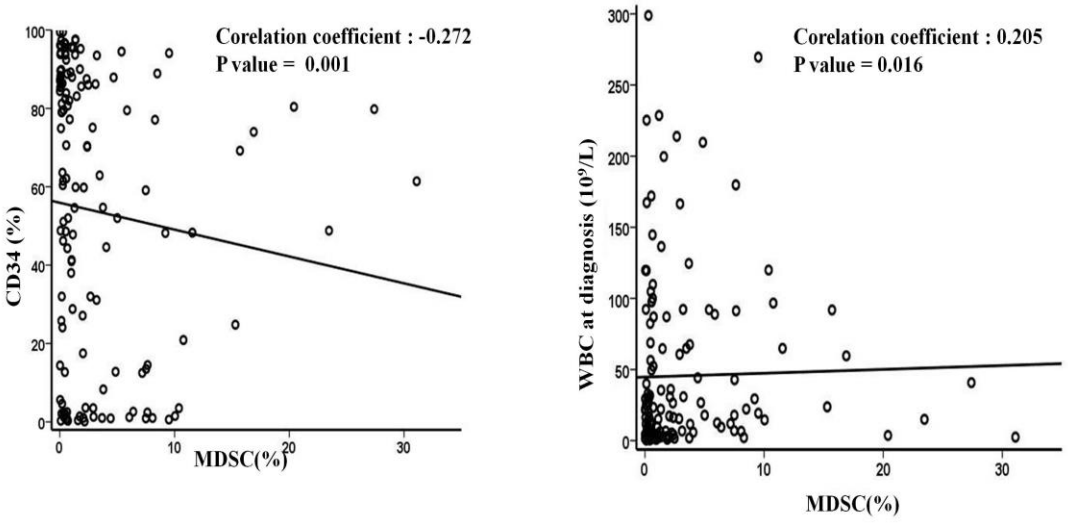


Figure 40. Graph showing correlation of MDSC percentage with CD34 percentage and total WBC counts at diagnosis. *Significant negative correlation of MDSC percentage with CD34 percentage (p=0.001) is noted, and a weak but significant positive correlation with WBC counts at diagnosis (p=0.016).*

Table 11: Comparison of conventional risk factors among cases in the highest quartile of MDSC expression (>3.74%) versus the rest in newly diagnosed AML. *p-value < 0.05 was considered significant.*

Variable	**MDSC > 3.74%** **N=34** **n(%)/ median(range)**	**MDSC ≤ 3.74%** **N=103** **n(%)/ median(range)**	**P-value**
Age (years)	39 (2-76)	42 (1-78)	0.67
WBC ($x10^9$/Lt)	25.2 (2.2-269.6)	15.4 (0.6-298.9)	**0.04**
CD34 (%)	46 (0.6-94.5)	70 (0.1-99.5)	**0.009**
Blast index	3.3 (0.03-256.12)	1.3 (0.01 -225.3)	0.83
CTG high risk group	3 (8.8%)	13 (12.6%)	0.55
NPM1 positive	6 (17.6%)	6 (5.8%)	**0.034**
FLT3 positive	5 (14.7%)	5(4.9%)	**0.056**
NPM1 and FLT3 positive	5(14.7%)	2(1.94%)	**0.003**

4.4. Evaluation of antibody response in APL patients

4.4.1. Presence of anti-RARα antibodies in APL patients

The GST and GST-RARα recombinant proteins were purified and quantified, and the concentration of GST is 2.5μg/μl and that of GST-RARα is 2μg/μl. The presence of the proteins was confirmed by immunoblotting against RARα antibodies (figure 41a).

Newly diagnosed APL patients (n=41) were screened for the presence of anti-RARα antibodies by ELISA, and the specific absorbance was calculated (explained in detail in methodology section). The median specific absorbance (SA) of APL patients was 0.638 (0.334-1.076) when compared with controls (n=14) whose SA was 0.347 (0.219-0.568). At maintenance 6 (n=18) the median SA was 0.596 (0.305-1.312). There was no statistical significance between the median SA of serum samples (n=18) available both at diagnosis (0.565 range: 0.312-1.06) and at maintenance 6 (0.595 range: 0.305-1.312) as illustrated in Figure 41b and 41c.

4.4.2. Confirmation of presence of anti-RARα antibodies in long-term follow up patients

The specificity of the anti-RARα antibody response was shown by western blots using recombinant GST or GST-RARα proteins with either anti-RARα antibody or patient serum (1-year post treatment) confirming the presence of RARα antibodies in the serum as shown in Figure 41d.

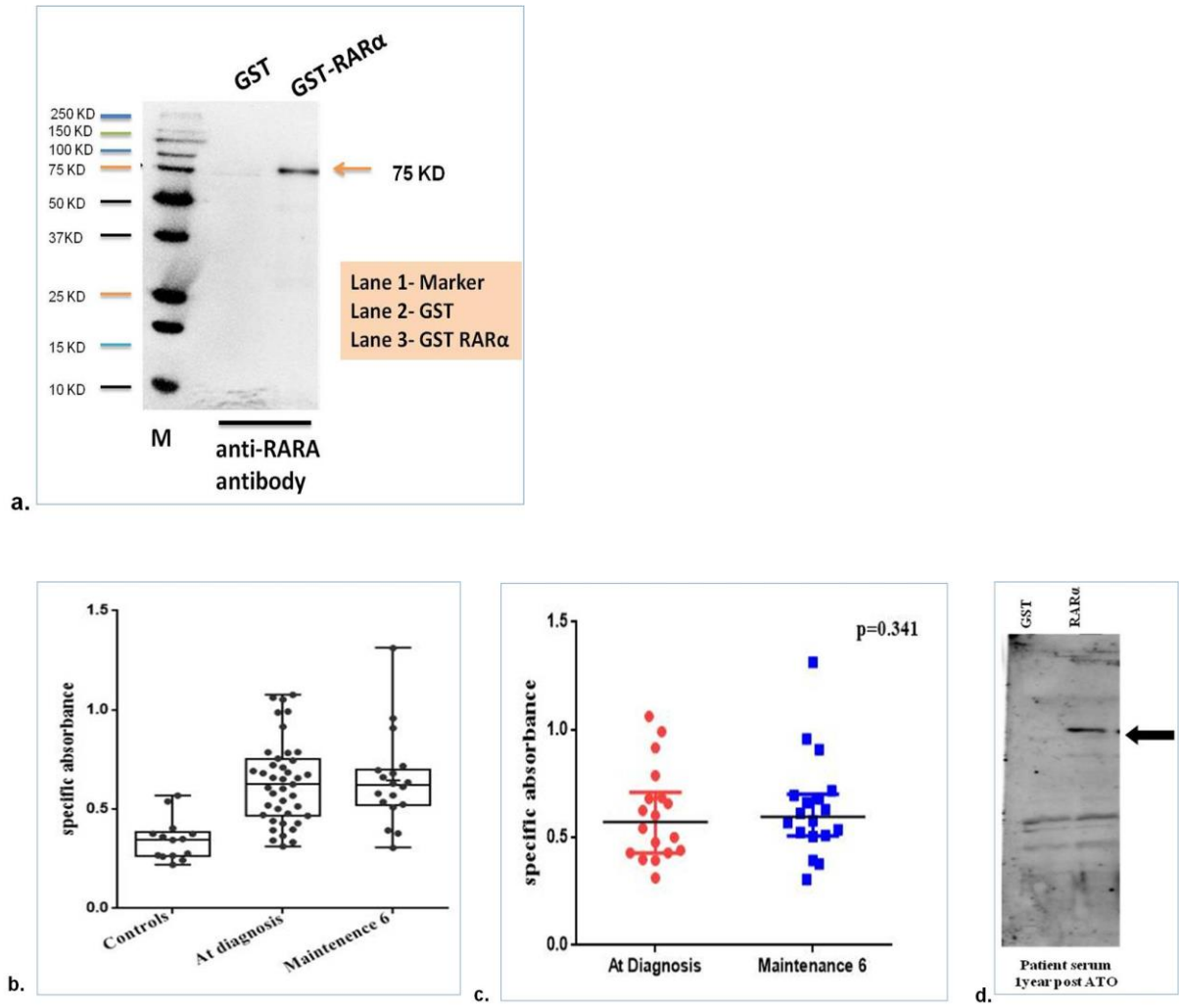


Figure 41. Presence of anti-RARα antibodies in serum of APL patients*. a. Western blots probed with an anti-RARα antibody (Santa Cruz, clone C20) (GST -25 kDa and GST-RARα- 75KDa) b. The graph showing the specific absorbance (SA) values of healthy controls (n=14) APL patients at day 0 (n=41) and serum samples collected during maintenance 6th cycle (n=18) and were screened for the presence of anti-RARα antibodies by ELISA. The specific absorbance was calculated by the formula, SA = $A_{RARα} - A_{GST}$. Each sample was tested in duplicates. c. Graph showing the SA of serum samples (n=18) available both at diagnosis and at maintenance 6. There was no statistical significance between the two groups. d. Representative immunoblot showing the presence of anti-RARα antibodies when probed with patient serum one-year post ATO treatment.*

4.5. Effect of arsenic trioxide on the modulation of immune cells

4.3.2.1. Determination of *in vitro* cytotoxicity of ATO towards myeloid and lymphoid cell lines

The IC50 of ATO towards all the cell lines used in this study was determined by MTT cell viability assay. There was a wide variation in the IC50 values between different myeloid and lymphoid cell lines. In K562 and Jurkat E6.1, the 50% inhibition concentration was not achieved at the concentrations used in this assay (>6 μM).

The mean IC50 values of ATO at μM concentrations of NB4 naïve cell line was 0.94± 0.04 μM, APL resistant cell line NB4EAsR1 was 3.13± 0.25 μM, UF1cell line (4.1± 0.3 μM), U937 (4.03± 0.15 μM), HL60 (2.9± 0.1 μM) and SUP-B15 was 1.3± 0.5 μM. All were done in triplicates and the IC50 values were generated using GraphPad prism software.

4.5.2. *In vitro* cytotoxicity of ATO on peripheral blood mononuclear cells

In vitro cytotoxicity of ATO on PBMNCS (n=3) revealed that IC50 was not achieved at the concentrations of ATO used in this assay (Figure 42). This confirms that ATO does not kill lymphocytes at the concentrations used for downstream experiments.

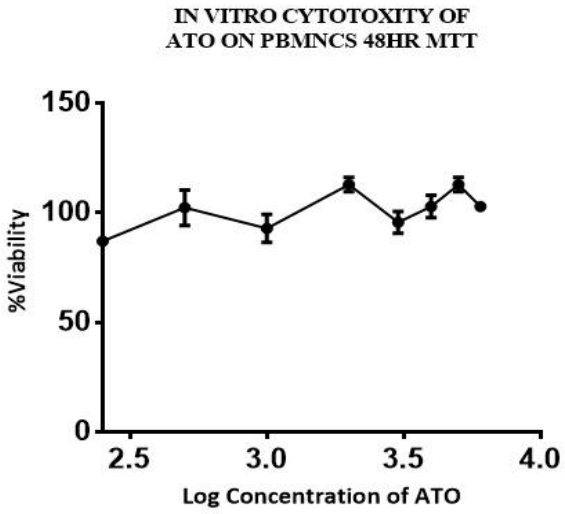


Figure 42. In vitro cytotoxicity of ATO on PBMNCs at 48 hours determined by MTT assay and IC-50 was calculated using GraphPad Prism software*. The experiment was done in triplicates.*

4.5.3. NK92MI cell line characterization

NK92MI cell line was characterized for its surface markers and receptors and were positive for CD2, CD7, CD11a, CD28, CD45, CD56, Nkp44, Nkp46, Nkp30, NKG2D, KIR2DL1, KIR3DL1/DL2, KIR2DS4, CD94 and CD226 and negative for CD1, CD3, CD4, CD5, CD8, CD10, CD14, CD16, CD19, CD20, CD34, HLA-DR and KIR2DL2. The histograms of NK receptor expression are illustrated in figure 43.

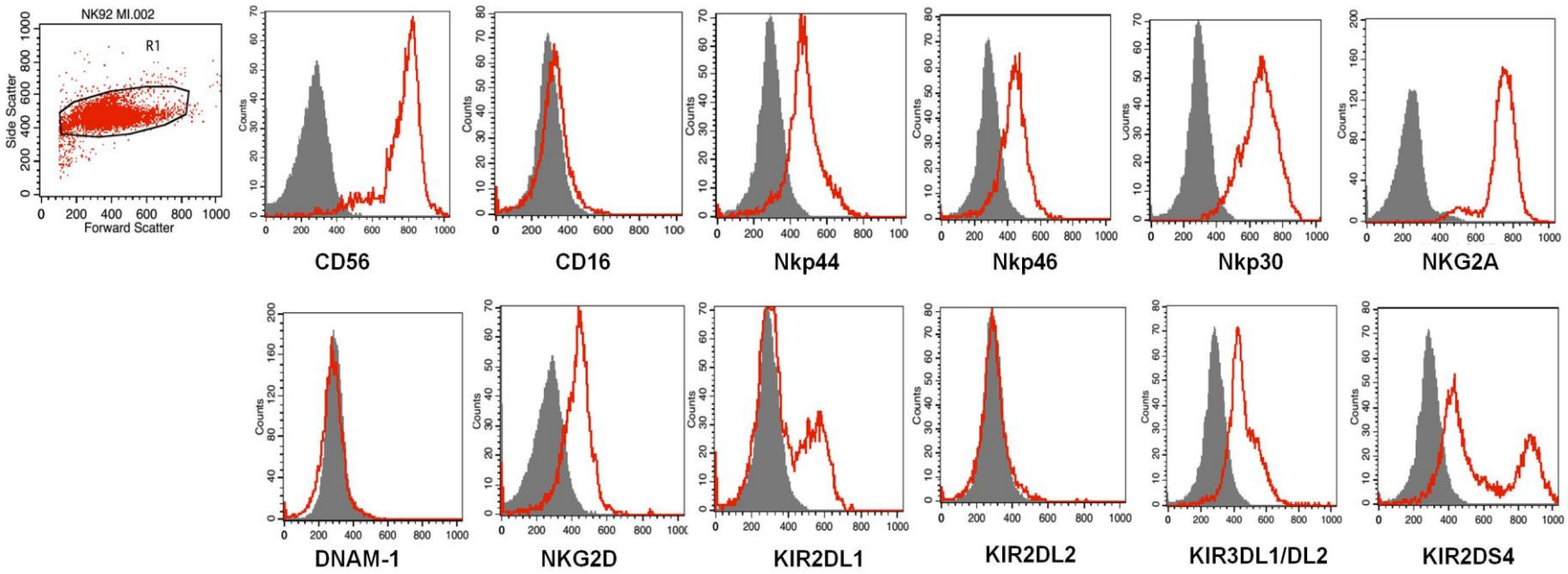
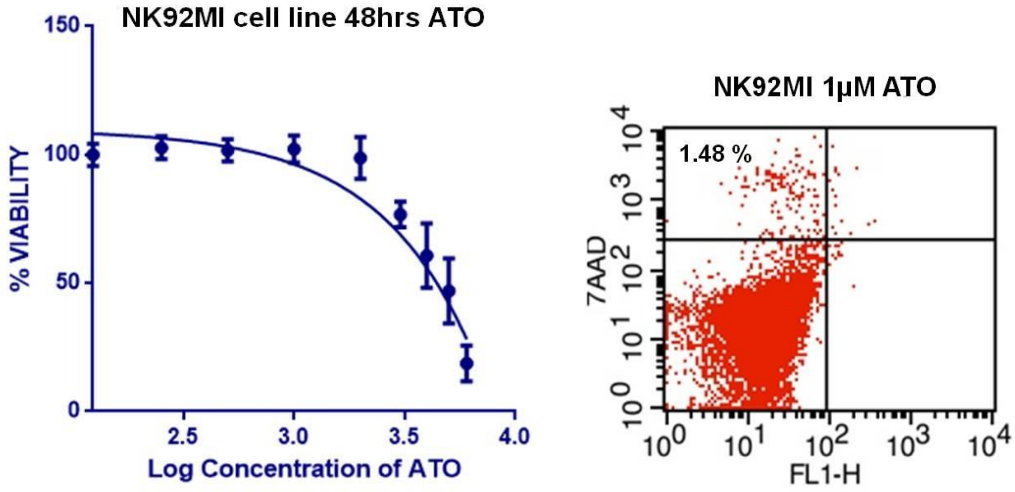


Figure 43: Representative histogram plots showing the expression of NK receptors in NK92MI cell line*. The shaded region represents the isotypic controls and the red line represents the positivity of each CD markers expressed on the cell line.*

4.5.4. Cytotoxic effect of ATO on NK92MI cell line

To make sure that ATO is not cytotoxic to the NK92MI cell line at the concentrations we used for the downstream experiments, we have done an MTT assay and a cell viability assay. The IC50 of NK92MI cell line was 3.84±0.3µM (n=4), and the 7AAD positive cells were 1.48% when treated with 1µM ATO for 24 hrs. Thus ATO has no direct cytotoxic effect on NK cell line (Figure 44).



a) Generation of IC-50 of ATO in NK92M1 cell line

b) Cell viability assay using 7AAD

Figure 44.Cytotoxic effect of ATO on NK92MI cell line measured by MTT assay and by 7AAD*. a) Graph representing the in vitro sensitivity assay of NK92MI cell line towards increasing concentration of ATO (post 48 hours incubation) using an MTT assay. The IC50 was generated using graph pad prism software (n=4). IC50 of NK92MI cell line was 3.8±0.3µM. b) The dot plot showing 1.48% cells positive for 7AAD when NK92MI cell line treated with 1µM ATO for 24 hours.*

4.5.5. Effect of ATO on proliferation of NK cells

We have also checked the proliferation of NK cell line when treated with 1μM ATO for 24hrs, 48 hrs and 72hrs. We have observed that there is no change in the rate of proliferation of this cell line when treated with ATO when compared to untreated as shown in Figure 45.

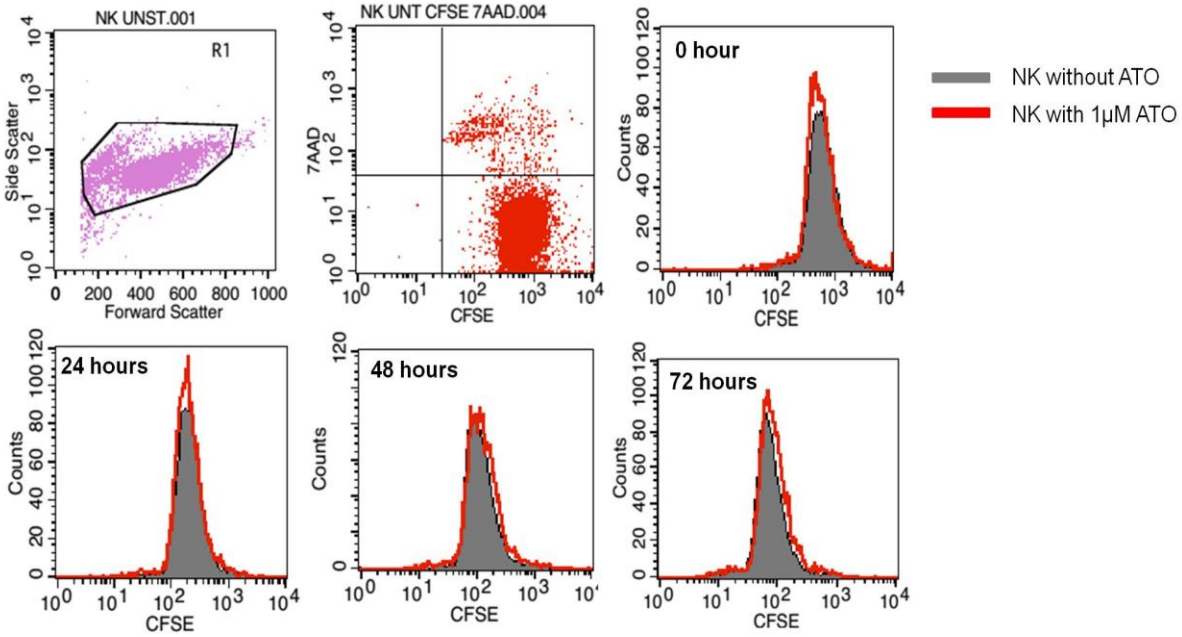


Figure 45. Representative histogram plots showing the proliferation of NK92MIcell line at 24, 48 and 72 hours by measuring the reduction in intensity of CFSE. *The shaded region represents the intensity of CFSE of NK cell line untreated and the red line indicated the intensity of CFSE of NK cell line treated with 1μM ATO.*

4.5.6. Cytolytic activity of NK cell line towards leukemic cell lines

Cytolytic activity of NK92MI towards different myeloid and lymphoid cell lines was assessed by CFSE 7AAD cytotoxicity assay. At the highest effector (NK cells): target (leukemic cell line) ratio of 10:1, the mean cytolytic activity against NB4 (n=3) was 21.5 ± 3.7%. Significant cytolytic activity was noted against K562 cell line. The percentage cytolytic activity of NK cell line towards 1μM ATO-resistant NB4EVAsR1 was only 4.4±0.65%. The mean cytolytic activity of NK towards all the myeloid and lymphoid cell lines was summarized in Figure 46 and Table 12.

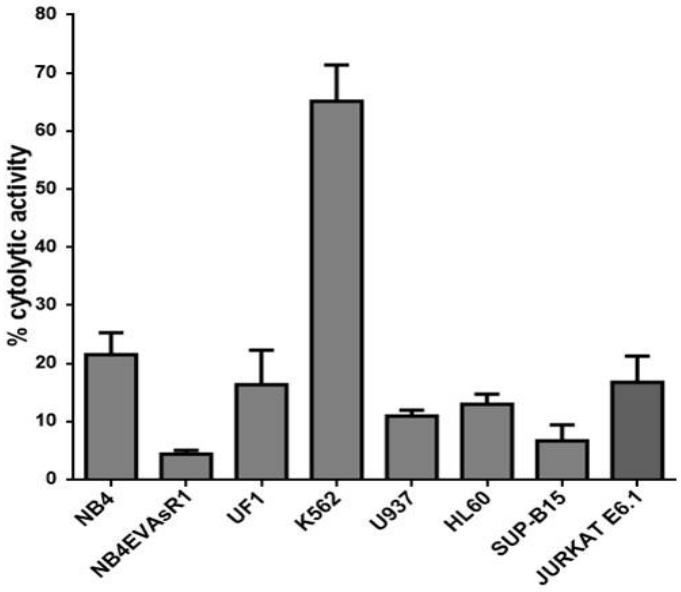


Figure 46. Bar diagram showing the mean percentage cytolytic activity of NK92MI cell line towards different myeloid and lymphoid cell lines (n=3) at an effector to target ratio of 10:1.

Table 12. The percentage cytolytic activity of NK92MI cell line towards different leukemic cell lines represented as mean ± SD (n=3).

Cell lines	% cytolytic activity (mean ± SD) (n=3)
NB4	21.5 ± 3.7
NB4EVAsR1	4.4 ± 0.65
UF1	16.32 ± 5.9
K562	65.15 ± 6.2
U937	10.9 ± 1.03
HL60	12.97 ± 1.7
SUP-B15	6.65 ± 2.7
Jurkat E6.1	16.7 ± 7.8

4.5.7. Role of ATO in enhancing the cytolytic activity of NK cells

Since cytolytic activity of NK cells was observed against NB4, we next checked the effect of ATO on the NK cytotoxic activity. We observed that NB4 when treated overnight with 1µM ATO (>99% viability retained after this exposure) significantly increased the cytotoxic effect of NK92MI cell line at all E: T ratios evaluated (p=0.002) with a mean percentage cytolytic activity of 28.2 ± 6.3% (n=5) at the highest E:T ratio of 10:1 as illustrated in Figure 47. No other cell line showed a similar increase in cytotoxic effect following exposure to ATO at these concentrations.

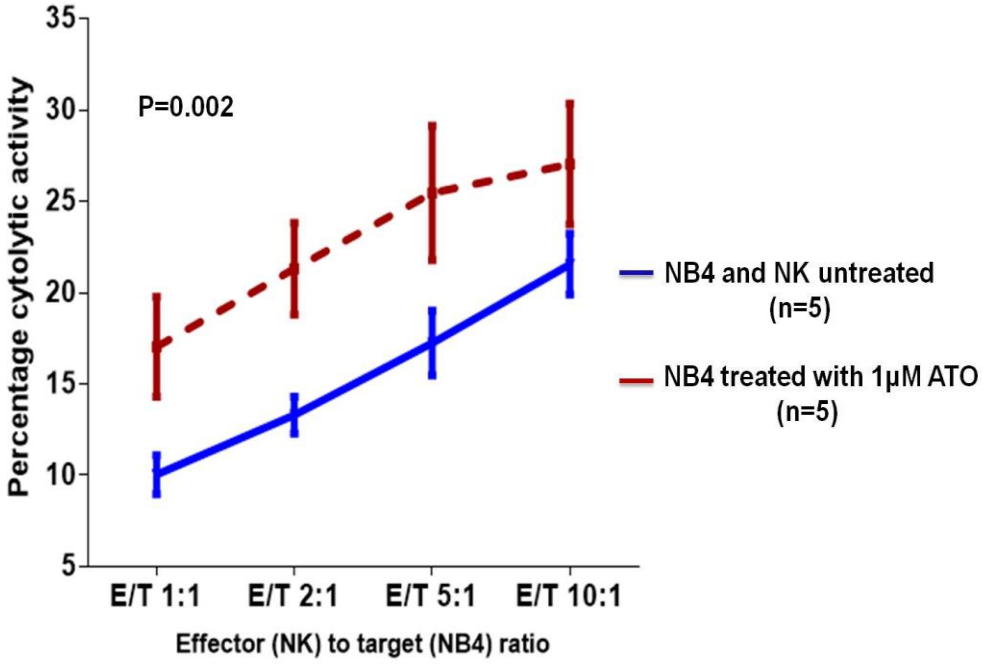


Figure 47. Comparison of percentage cytolytic activity of NK92MI cell line towards NB4 untreated and treated with ATO (1µM) for 12 hours*. The values were plotted as mean with SEM (n=5).*

4.5.8. Cytolytic activity of NK cell line assessed by degranulation assay

To check the effect of ATO on NK cell activity, CD107a degranulation assay was done using NK92MI cell line as effector and targets were NB4, NB4EVAsR1, UF1, K562, U937, HL60 and Jurkat cell lines. At an E: T ratio of 2:1 (n=3), the mean percentage of CD107a with NB4 cell line was 20.4±1.8% at 5 hours whereas with NB4EV-AsR1 was 5.08± 0.8%, UF1 (2.33±0.33%), K562 (31.3± 1.6%), U937 (35.6±4.8%), HL60 (4± 1.13%) and Jurkat was 4.2± 0.5%. On treatment with 1µM ATO

for 12 hours there was a significant increase in the percentage of CD107a with NB4 cell line (33.6 ±3.8 %) which was not seen with other cell lines on treatment (Figure 48-49). This infers that there is an increased cytotoxic activity of NK cell line towards NB4 on treatment with ATO.

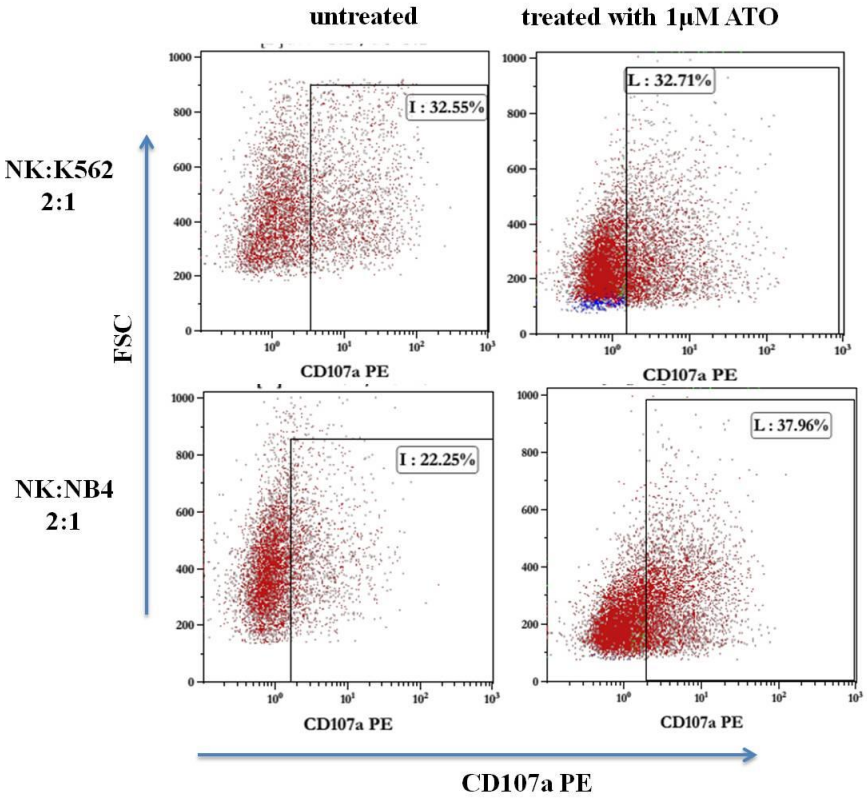


Figure 48. Histogram plots showing the CD107a percentage when NK and K562 or NK and NB4 were cocultured at the ratio of 2:1 for 5 hours (n=3).

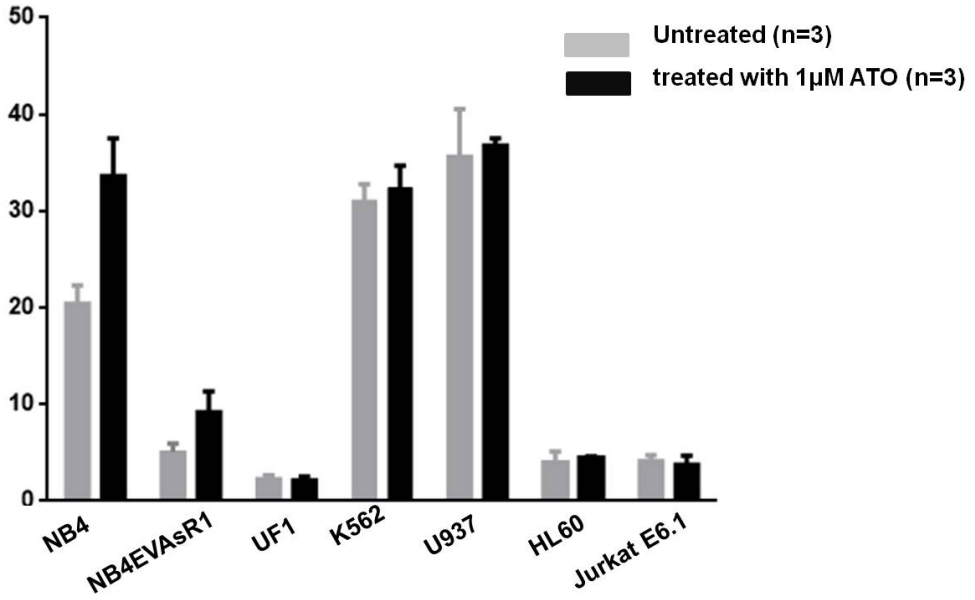


Figure 49. Bar graphs showing the percentage expression of CD107a when NK cell lines are cocultured with leukemic cell lines at the ratio of 2:1 for 5 hours*. The grey bars represent the experiment with untreated leukemic cell lines and the black bars represent the experiment after exposure of leukemic cell lines to 1uM ATO for 12 hours(n=3).*

4.5.9. Role of ATO in altering NK cell line receptor profile

The effect of ATO in altering the NK cells receptor profile was then evaluated. Exposure of NK92MI cell line to 1μM ATO (non-cytotoxic dose: IC50-3.8μM) for 6 hours resulted in increased expression of activating receptors NKG2D, NKP30 and KIR2DS4 and inhibitory receptor NKG2A and decrease in inhibitory receptors KIR3DL1/DL2. The cells also retain 99% viability at the end of 24 hours by 7AAD assay. There were no changes in the expression of NKP46, KIR2DL1, KIR2DL2 and DNAM1

receptors**.** There was no further increase in the expression of the above markers when treated with increased concentration (2μM ATO) or with increased exposure time (24 hours). Figure 50 shows the upregulation of activating and inhibitory receptors on NK92MI cell line following treatment with 1μM ATO for 6 hours.

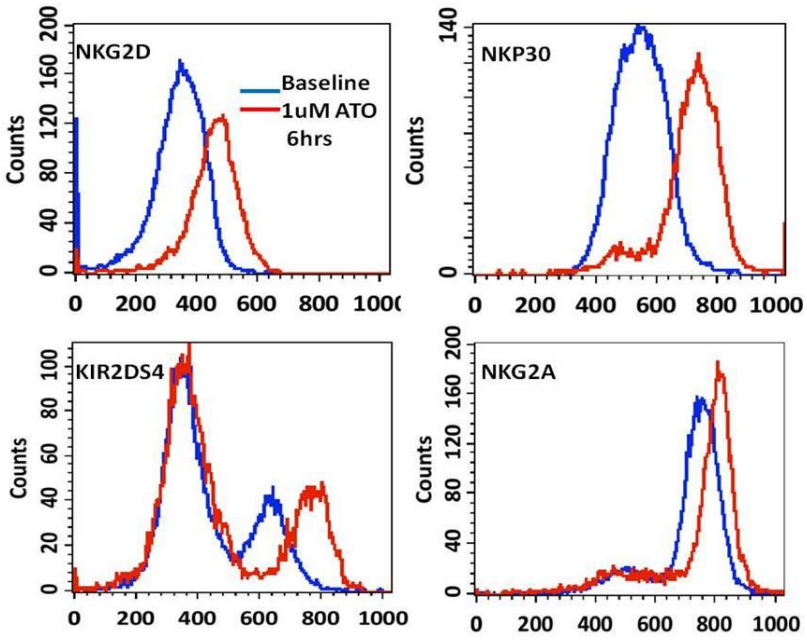


Figure 50: Representative histogram plots of NK activating receptors NKG2D,NKp30 and KIR2DS4 and NK inhibitory receptor NKG2A of NK cell line treated with 1μM ATO for 6 hours (n=5). *The blue lines represent the baseline expression and the red lines which show a shift towards right indicated the increased expression with 1μM ATO treatment for 6 hours.*

4.5.10. Expression of NK ligands induced by ATO in NB4 cell line

We next evaluated the effect of exposure of leukemic cell lines to 1µM and 2µM ATO for 6 hours and 24 hours on NK ligand expression by flow cytometry. There was a baseline expression of MICA/B in NB4, U937, K562, NB4EVAsR1, Jurkat and SUP-B15 cell line. However, there was a significant increase in activating ligand MICA/B only in NB4 cell line (n=3; P=0.016) at 1µM ATO for 6 hours which was not seen in any of the other cell lines. Figure 51 shows the induction of NK activating ligands MIC A/B on different cell lines on treatment with 1µM ATO for 6 hours. Similar significant increased expression of CD112/Nectin-2 (DNAM-1 ligand) and HLA Class I was seen in NB4 as shown in Figure 52. At the concentration and duration of ATO exposure used in these experiments, there was no significant cytotoxicity in any of the cell lines. There was no further increase in the expression of the above ligands when treated with increased concentrations or with increased exposure time.

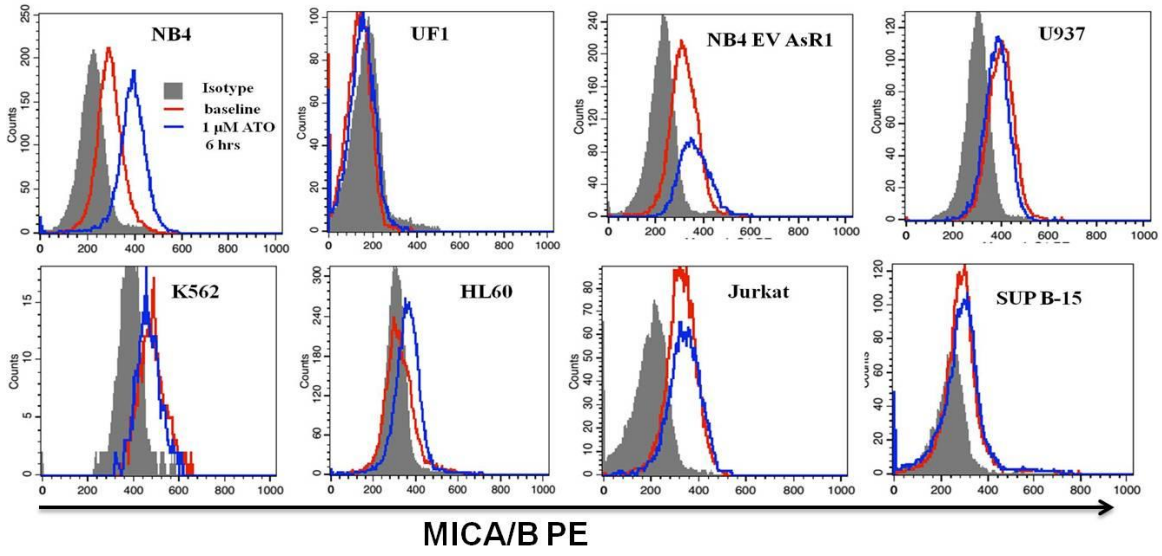


Figure 51: Representative histogram plots of MICA/B expression in myeloid and lymphoid cell lines treated with 1µM ATO for 6 hours (n=3). *The shaded region represents the isotypic controls and the red line represents the baseline expression of ligands. The blue lines which show a shift towards the right in NB4 indicating increased expression of MICA/B after ATO treatment which was not observed in other cell lines.*

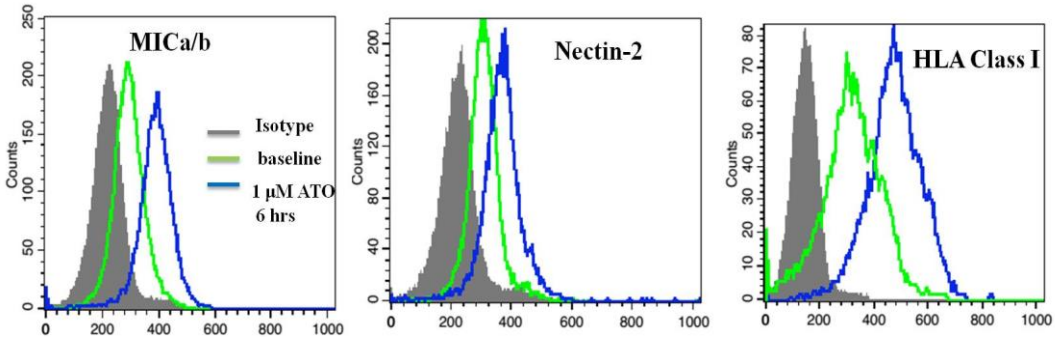


Figure 52. Representative histogram plots of Nectin-2, MIC A/B, and HLA Class I NK activating and inhibitory ligands on NB4 treated with 1µM ATO for 6 hours(n=3). *The shaded region is the isotypic controls, and the green line represents the baseline expression of all ligands. A shift towards the right in all plots (blue lines) shows an increase in expression of ligands after ATO treatment.*

4.6. Genotype and haplotype analysis of activating and inhibitory KIR genes

KIR genotyping was done on 55 patients with APL who received treatment with single agent ATO based regimen. The median follow-up of this cohort was 46 months and 16 cases relapsed following initial therapy. All the 16 KIR genes (6 activating receptors KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR3DS1 and 8 inhibitory receptors KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL4, KIR2DL5, KIR3DL1, KIR3DL2, KIR3DL3) and 2 pseudo genes (KIR2DP1, KIR3DP1) have been screened in all the patients (Table 13).

Out of the 55 patients screened, 18.2% (n=10) of the patients have A haplotype and 81.2% (n=45) patients have B haplotype. Briefly, haplotype A is defined by the presence of KIR2DL1, KIR2DL3, KIR3DL1, KIR2DS4 along with framework genes *(*KIR2DL4, KIR3DL2, KIR3DL3 and KIR3DP1) and haplotype B by the presence of one or more genes encoding activating KIRs, KIR2DS1/2/3/5, KIR3DS1 and inhibitory KIRs, KIR2DL5A/B and KIR2DL2. KIR2DL1, KIR2DL4, KIR3DL2, KIR3DL3 and the two pseudo genes were present in all patients.

There was no specific association with any specific genotype or haplotype with the risk of relapse or any other clinical outcome parameter. However, there was a trend towards significance in the presence of KIR2DL2 inhibitory receptor in relapse patients (13 relapse patients out of 16 are positive for KIR2DL2) when compared to non-relapse

group (p=0.069) with a hazard ratio of 3.2 (95%CI: 0.91-11.26). Table 14 shows the presence or absence of KIR genes screened in 55 patients and the Figure 53 shows the representative image of haplotypes in healthy controls and patients.

Table 13: **Table showing the presence or absence 16 KIR genes screened in APL patients (n=55)**. The framework genes *KIR2DL4, KIR3DL2, KIR3DL3* and *KIR3DP1, the pseudogenes KIR3DP1 and KIR2DP1 and KIR2DL1 is present in all the patients and controls*

KIR genes	Number of APL patients (n=55)	
	positive	negative
2DL1	55	
2DL2	34	21
2DL3	44	11
2DL4	55	-
2DL5 A/B	40	15
2DS1	33	22
2DS2	32	23
2DS3	27	28
2DS4 Del	42	13
2DS4 Ins	19	36
2DS5	32	23
3DL1	45	10
3DL2	55	-
3DL3	55	-
3DS1	32	23
2DP1	55	-
3DP1	55	-

.

Table 14. Univariate analysis of the presence of KIR genes on relapse-free survival.

KIR genes	relapse (n=16)		non relapse (n=39)		RFS HR(95%CI)	p value
	positive	negative	positive	negative		
2DL1	16	0	39	0	-	-
2DL2	13	3	21	18	3.2(0.91-11.26)	0.069
2DL3	13	3	31	8	1.2(0.34-4.17)	0.789
2DL4	16	0	39	0	-	-
2DL5 A/B	12	4	28	11	1.2(0.38-3.66)	0.775
2DS1	10	6	23	16	1.2(0.42-3.19)	0.774
2DS2	12	4	20	19	2.6(0.84-8.06)	0.098
2DS3	11	5	16	23	2.5(0.85-7.06)	0.097
2DS4 Del	6	10	30	9	0.8(0.25-2.42)	0.664
2DS4 Ins	6	10	13	26	1.2(0.45-3.38)	0.692
2DS5	13	3	23	16	0.9(0.34-2.45)	0.855
3DL1	13	3	32	7	1.0(0.29-3.55)	0.987
3DL2	16	0	39	0	-	-
3DL3	16	0	39	0	-	-
3DS1	10	6	22	17	1.3(0.47-3.53)	0.631
2DP1	16	0	39	0	-	-
3DP1	16	0	39	0	-	-
Haplotype A/B	3A and 13B		7A and 32B		1.0(0.29-3.62)	0.961

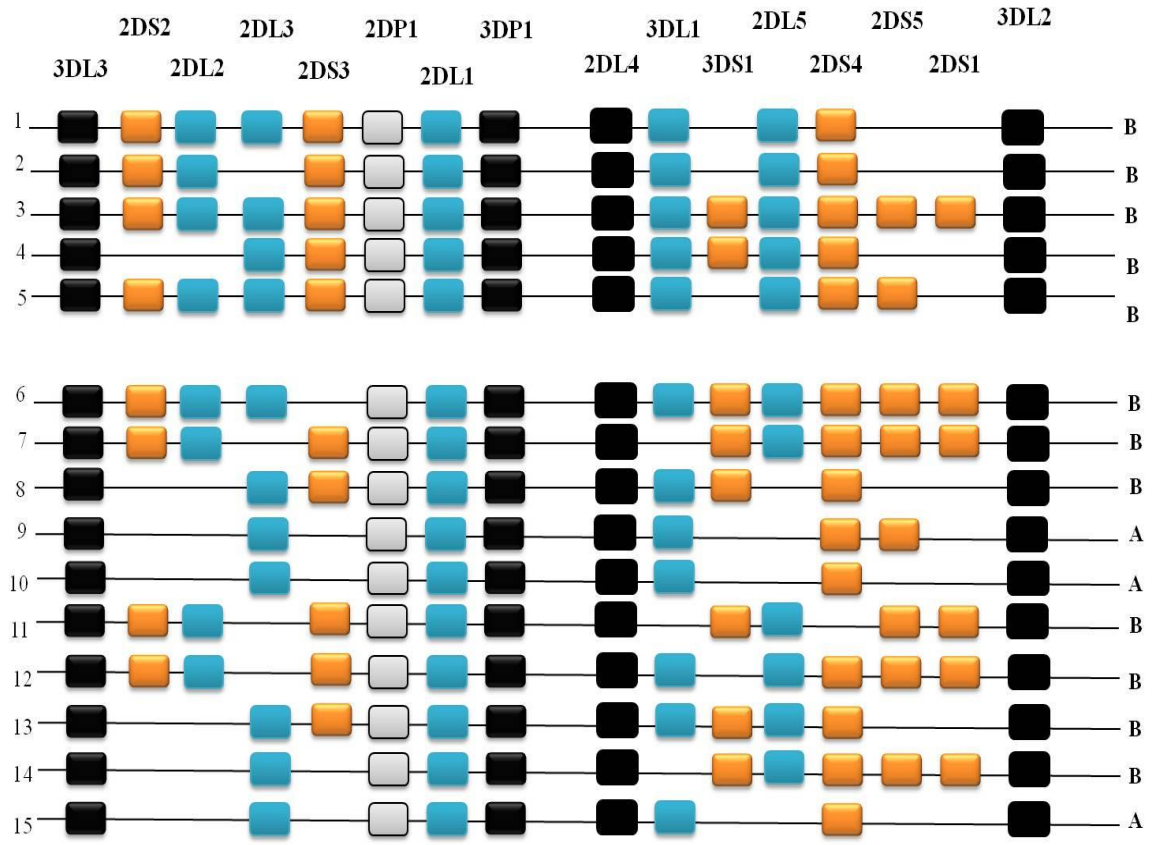


Figure 53: Representative image of KIR genes and haplotypes in control samples (1-5) and patients (6-15). *Haplotype A is defined by the presence of KIR2DL1, KIR2DL3, KIR3DL1, and KIR2DS4 along with framework genes and haplotype B by the presence of one or more genes encoding activating KIRs, KIR2DS1/2/3/5, KIR3DS1 and inhibitory KIRs, KIR2DL5A/B and KIR2DL2. KIR2DL1, KIR2DL4, KIR3DL2, KIR3DL3. Out of the 55 patients screened 10 were of haplotype A and 45 with haplotype B.*

4.7. Assessment of NK differentiation from CD34 cells with and without ATO treatment

Since we observed a delay in the NK cell recovery even after 6 months post treatment, we planned to investigate the effect of ATO on CD34 differentiation *in vitro*. Starting with 1.1% $CD34^+$ cells in cord blood sample, after 1st sorting it was enriched to 49.3% and 95.3% purity was obtained after second sorting (Figure 54). Sorted CD34 cells were characterized by flow cytometry and were seeded in a 24 well plate at a concentration of $0.3x10^6$cells/ml and media was replenished for every 3 days. In another set of experiments sorted $CD34^+$ cells were seeded in media containing 0.5 μM ATO. The cell counts were checked on day 8 and day 14 and differentiation were evaluated by flow cytometry. The cell counts on day 8 and day 14 of CD34 cells were 1.5 ± 0.26 $x10^6$ cells/ml and 2.8 ± 0.8 $x10^6$ cells/ml respectively (n=3) whereas it was lower in cells treated with ATO with a cell concentration of 0.9 ± 0.3 $x10^6$ cells/ml and 1.06 ± 0.5 $x10^6$ cells/ml on day 8 and day 14 respectively.

NK differentiation was checked on day 8 and day14. Flow cytometry data showed 5.1±0.3 % cells were positive for $CD56^+CD3^-$ on day 8 and it increased to 7.7±0.4% on Day14 in the culture without ATO. Whereas CD34 seeded in media with ATO had only 2.3±0.4% $CD56^+CD3^-$ cells on day 8 and 5.2±0.4 % on day 14 (n=3) showing the delay in NK differentiation (Figure 55).

We also looked at the T cells, B cells and myeloid compartments on day 14. There is a slight increase in the myeloid compartment (81.6±5.5%) on day 14 in culture with ATO when compared with untreated culture (77.8±5.9%). CD3 and CD19 in untreated culture (16.8±3.4% and 1.6±0.7% respectively) was similar to the treated culture (17.4±5.2% and 0.8±0.3% respectively) (n=3) as represented by the pie chart (Figure 56).

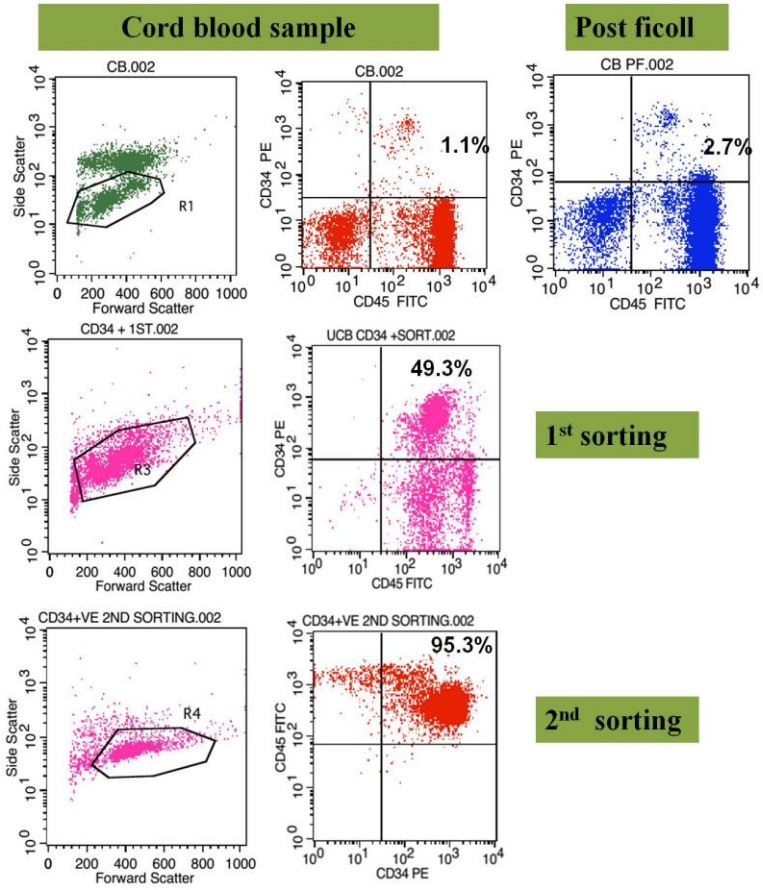


Figure 54: The representative dot plot images showing the percentage CD34⁺cells in cord blood pre and post sorting. *The post sorted cells were again subjected to one more round of sorting using an Easy Sep magnet and the purity obtained was 95.3%.*

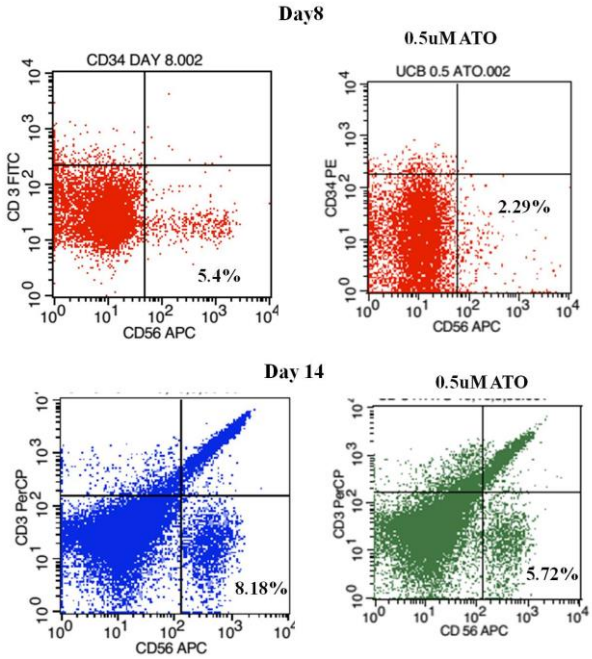


Figure 55: Representative dot plots showing the percentage of differentiated NK from CD34+ cells (untreated or treated with 0.5µM ATO) on Day8 and Day14.

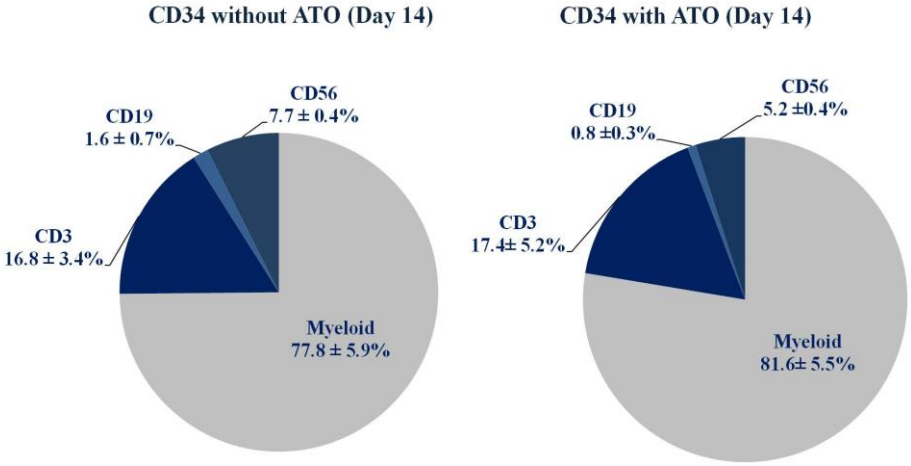


Figure 56: Pie chart showing the percentage of T cells, B cells, NK cells and myeloid differentiated from CD34+ sorted cells with or without 0.5µM ATO on day 14 (n=3).

4.8. Role of transcription factors involved in NK cell differentiation

Since there is a reduction in NK cell number and differentiation on exposure to ATO, we evaluated the role of transcription factors involved in the differentiation of CD34 cells to mature activated NK cells. It has been proposed that the transcription factors play a significant role in the different maturation stages of NK, but the role of ATO in NK transcription profiling is less explored.

RNA was extracted from CD34 sorted cells on day 0 and day 14 untreated and day 14 CD34 cells treated with 0.5μM ATO. Taqman based gene expression assay was performed to study the expression of NK transcription factors (TFs) Eomes, Ikzf2, Prdm1, Ets1, Tox, Klf4 and Tbx21 and ΔCT values were obtained by normalizing with GAPDH. Fold expression of CD34 on Day 14 and CD34 treated with ATO were calculated by 2^ΔΔCT method relative to CD34 on Day0.

We observed a significant decrease in the expression of TFs Ikzf2 , Ets 1 and Tox in Day 14 CD34 cells treated with ATO when compared to untreated (p=0.000, p=0.002 and p=0.002 respectively) ,which are shown to be involved in NK cell maturation. The expression of Prdm1 and Klf4 was also decreased even though not significant as shown in Figure 57. The expression of Tbx21 was increased on treatment with ATO at day14.

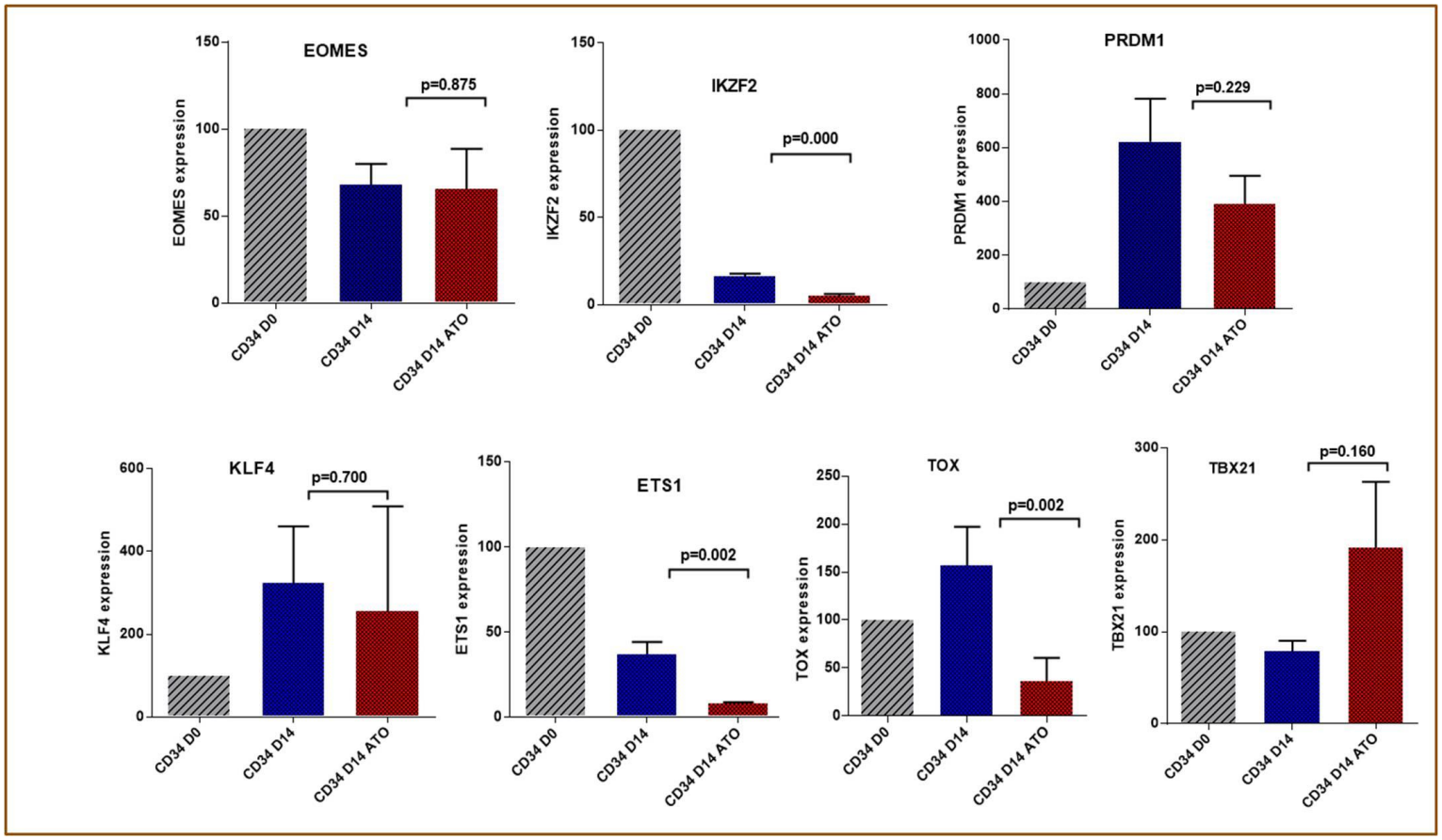


Figure 57: Expression of differentially expressed NK transcription factors determined by 2^ΔΔCT method. *The expression of TFs was normalized to GAPDH and the relative expression of CD34 day 14 and CD34 day 14 treated with ATO was calculated with CD34 Day 0 (n=3). p-value less than 0.05 was considered significant.*

4.9. Efficacy of PML-RARα targeted DNA vaccine in a mouse model of APL

4.9.1. Establishment of the preclinical animal model of APL

To evaluate the role of ATO in APL mouse model, we took advantage of the 'APL transplantable' mouse model and re-established this model in our laboratory by injecting the leukemic blasts obtained from the laboratory in France. 6 weeks old wild type FVB/N mice (n= 6) were injected intravenously in the tail with $5x10^4$ blasts cells. After one week, leukemia was confirmed by blood cell counts, morphology, organ pathology, RT-PCR for bcr1 transcripts and by immunophenotyping. The leukemic FVB/N mice without any intervention was mentioned as placebo throughout the study.

Leukemia was assessed by the appearance of high leukocyte and low platelet counts. The median WBC count of the APL FVB/N was $17.3x10^3/\mu l$ (9.6-22.1) and the median platelet count was $672x10^3/\mu l$ (480-793) (n=6). The bone marrow cytospin revealed more than 80% of immature myeloid cells. The spleen was significantly enlarged in leukemic mice when compared with the wild-type FVB/N. Qualitative nested PCR showed bcr1 positivity from the total RNA extracted from peripheral blood and splenic cells. Immunophenotype of peripheral blood from leukemic mice showed 23.5% of immature $Gr1^+CD117^+$ cells (Figure 58 1-4).

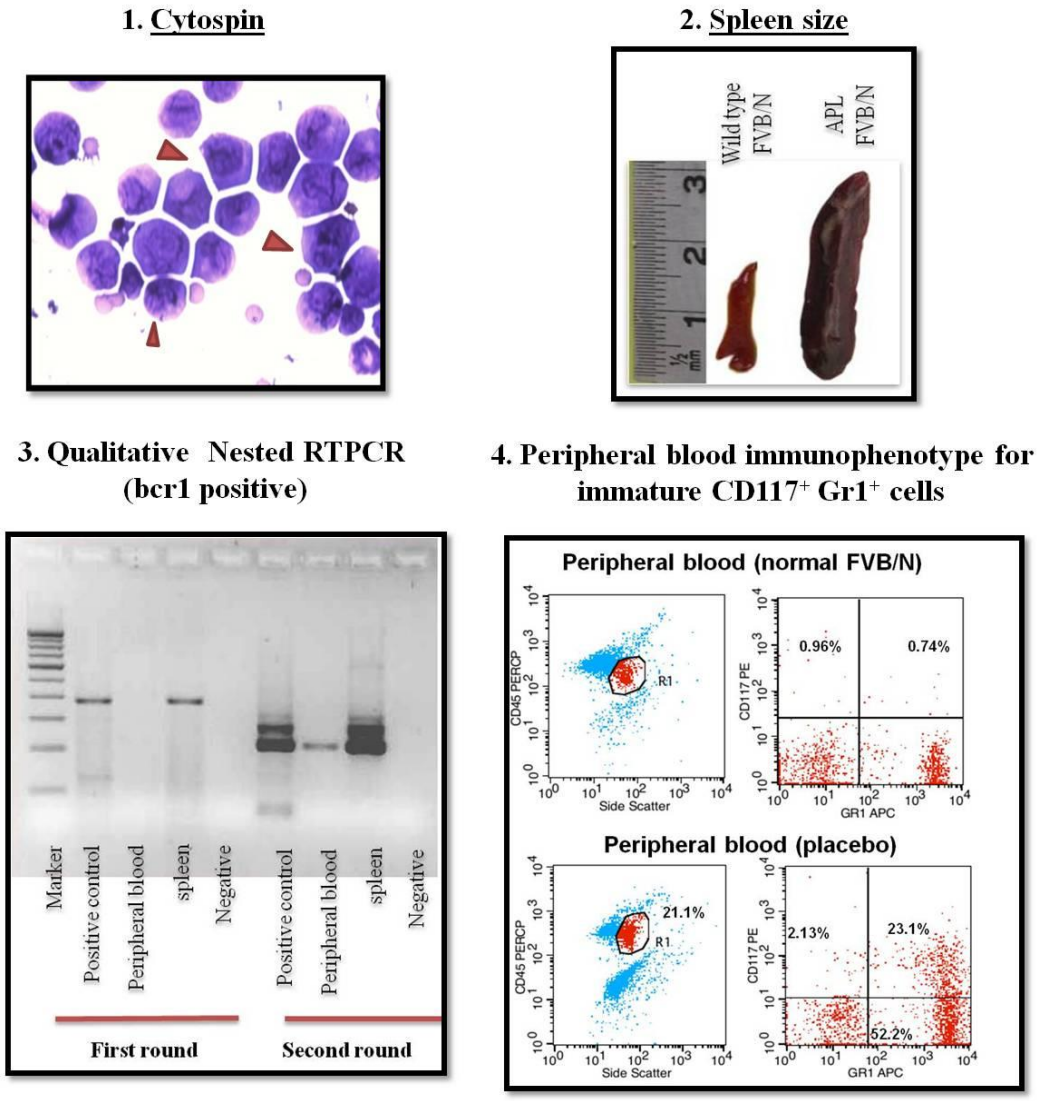


Figure 58. Assessment of leukemia in the preclinical mouse model of APL. *1. Bone marrow cytospin from leukemic FVB/N stained with Wright's Giemsa (magnification at 40x). Large pleomorphic cells showing inconspicous to prominent nucleoli with moderate cytoplasm (red arrow heads). 2. Spleens from normal mice and leukemic mice 3. RT-PCR analysis of PML-RARa fusion transcript in the peripheral blood and splenic cells of leukemic FVB/N. Representative result from nested pcr is shown. 4. Flow cytometry analysis of Gr1+CD117+ cells in peripheral blood of leukemic mice. All the experiments were assessed on day7 after the injection of APL blast cells.*

Survival analysis

The median survival of leukemic FVB/N without any interventions was 28 days (21-31 days) as represented by the Kaplan-Meier curve (Figure 59). After the confirmation of leukemia, the mice were sacrificed, and spleen and bone marrow cells were cryopreserved in -80^{0}C for further experiments.

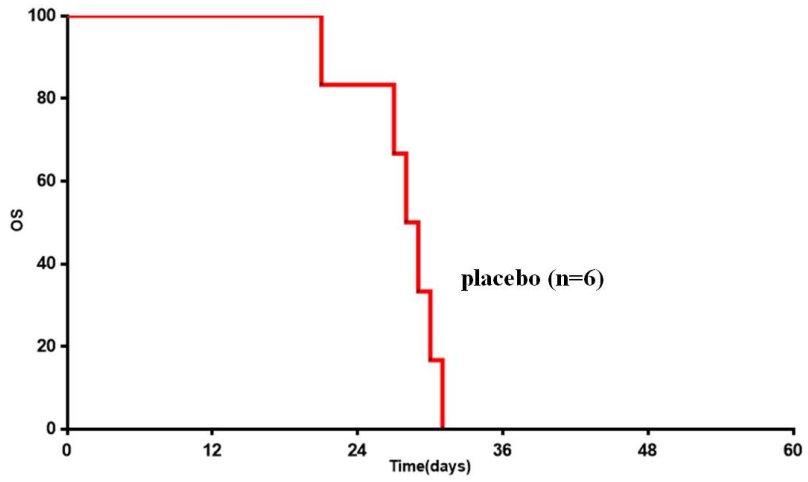


Figure 59: Kaplan Meier curve showing the survival of leukemic FVB/N mice without any intervention*. The median survival was 28 days (n=6).*

Establishment of preclinical animal model of APL treated with ATO

Using the above APL transplantable model we then designed a protocol that more closely resembled the human APL clinical setting in which ATO was initiated after leukemia had been fully established in the mice. 6 week old wild type FVB/N mice was injected intravenously in the tail with 5x 10^4 blasts and from Day 7 ATO was administered by daily intraperitoneal injection at the concentration of 5 µg/g mice for 28 days. Disease status will be monitored clinically and biologically in mice, and peripheral blood samples were collected by retro-orbital bleeding on day 20, day 35 and day 60. Leukemic burden was assessed by WBC and platelet counts, spleen size, the presence of PML-RARα transcripts and percentage of $Gr1^+CD117^+$ cells. H&E staining of bone marrow and spleen of placebo revealed that there was a diffuse infiltrate of immature cells replacing the marrow and spleen whereas in ATO-treated mice there was a reduction in the immature cell infiltrates. The spleen size was slightly reduced in ATO treated mice and the leukemic burden was reduced as shown by the immunophenotype of $CD117^+Gr1^+$ cells on day 20 (Figure 60). The median survival of ATO-treated mice is 45 days (33-48 days).

With this ATO mouse model, different interventions were done and samples were collected and monitored for survival (explained in detail in methodology section). Each set of experiments were mentioned as protocols.

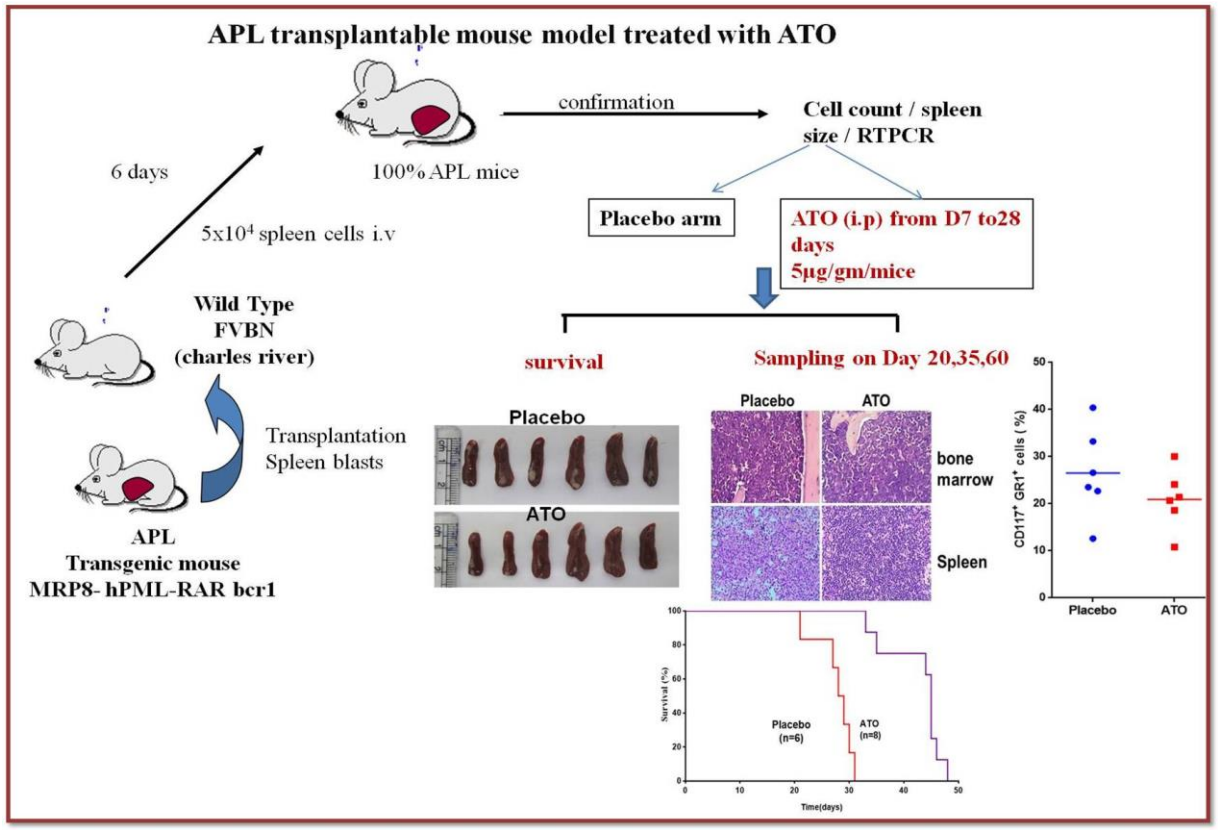


Figure 60: Schematic representation of preclinical APL transplantable mouse model treated with ATO. *ATO was administered at the concentration of 5 µg/g mice (i.p) from Day 7 for 28 days. Demonstration of leukemic burden reduction in the treated arm by a slightly reduced spleen size and H & E staining of bone marrow and spleen shows reduced immature cell infiltrates. Flow cytometry analysis shows reduced leukemic cells (CD117+ and Gr1+ cells) in bone marrow and the median survival of ATO-treated group is 45 days (33-48 days).*

4.9.2. Efficacy of DNA vaccine in combination with ATO and ATRA

Protocol 1

The leukemic mice (n=34) were separated into five therapeutic groups: placebo (n=6), ATO alone (n=8), ATRA alone (n=5), ATO+ATRA (n=7) and ATO+ATRA+DNA vaccine (n=8) and the interventions and time points of sampling were illustrated in Figure 61**.** The WBC and platelet count done on three sampling time points were shown in Table 15.

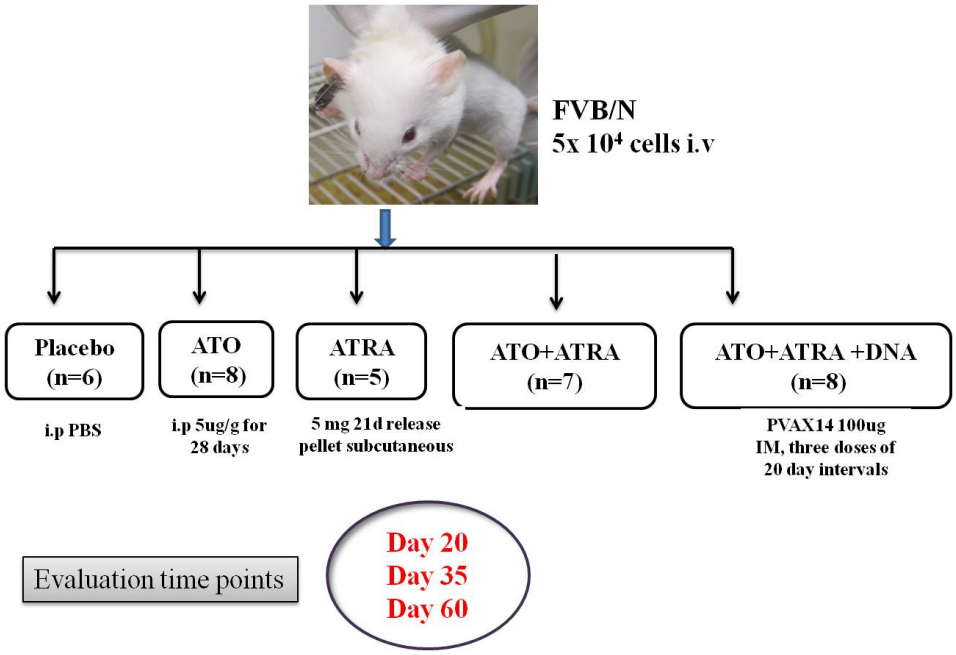


Figure 61: Schematic representation of Protocol 1. Efficacy of DNA vaccine in combination with ATO and ATRA*. ATO was administered daily by i.p at the concentration of 5 µg/g mice from Day 7 for 28 days. 5mg ATRA was administered by subcutaneous implantation of a 21-d release pellet). Control mice were given TBS (i.p). DNA plasmid vaccine was given intramuscularly at a dose of 100µg per mouse for 3 doses with 20-day intervals. Samples were evaluated on day20, day35 and day 60.*

Table 15. The median WBC counts and platelet count at Day 20, 35 and 60 in different experimental groups.

Groups and time points of sampling		WBC ($x10^9$/Lt) median(range)	Platelet ($x10^9$/Lt) median(range)
Placebo (n=6)	Day 20	17.3 (9.6-22.1)	672(480-793)
ATRA (n=5)	Day 20	29.2 (14.5-32.2)	854 (646-1292)
	Day35	12.6 (3.6-13.3)	976 (763-1176)
ATO(n=8)	Day 20	11.3 (8.1-21.1)	528 (341 -554)
	Day35	21.25 (10.7-54.9)	630 (510-946)
ATO+ATRA	Day 20	16.4 (8.6-25.4)	676 (606-986)
(n=7)	Day 35	7.45 (3.4-7.6)	857 (793-1508)
	Day 60	7.1 (4.8-10.2)	687 (440-796)
ATO+ATRA	Day 20	23.4 (17.2-26.6)	740 (606-895)
+DNA(n=8)	Day 35	7.15 (4.7-8.2)	879 (832-1044)
	Day 60	8.4 (8.3-10.2)	379 (373-650)

Spleen size

The mean spleen size of the placebo group was 2.82 ±0.29cm and that of ATRA was 2.5± 0.42cm and ATO was 2.7±0.25cm. There is a significant reduction in spleen size in the ATO+ATRA group (2±0.1cm) (p=0.002) and in ATO+ATRA+DNA group

(1.76±0.05cm) (p=0.001) when compared with the ATO group. The figure 62 below shows the pictorial representation of spleen sizes of different groups.

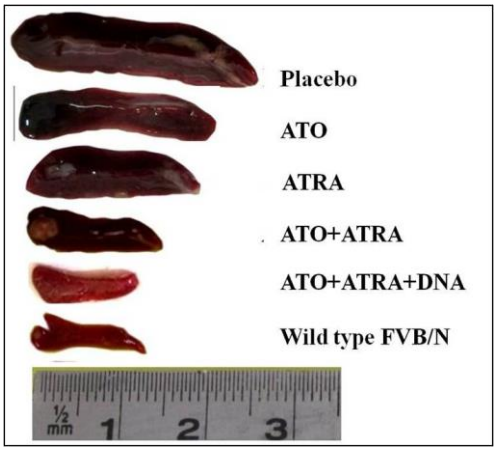


Figure 62: A representative figure showing the spleen sizes of different treatment groups when compared with wild-type FVB/N.

Survival analysis

When compared with placebo having a median survival of 28 (21-31) days, the median survival of ATO-treated leukemic FVB/N was 45 (33-48) days which was significant (p <0.0001). The median survival was significantly much higher in the other three groups with ATRA-treated mice having 56 (52-100) days (p=0.001), ATO+ATRA treated mice having 200 (31-200) days (p=0.002) and ATO+ATRA+DNA treated mice having a median survival of 200 (37-200) days (p=0.001). The combination of ATO and

ATRA along with DNA vaccine significantly extended the survival when compared to ATO or ATRA alone. The Kaplan-Meier curve shows the survival between various groups of FVB/N treated with the above mentioned combinations (Figure 63).

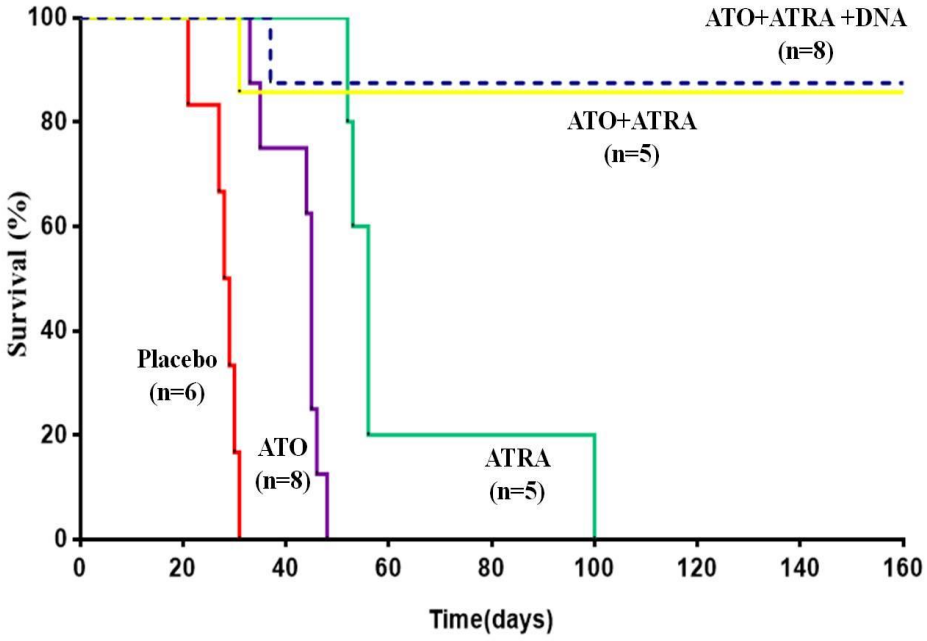


Figure 63: Kaplan Meier survival curves of APL FVB/N treated with ATO, ATRA, ATO+ATRA and ATO+ATRA+DNA vaccine

Protocol 2

In the next set of experiments, we have looked at the effect of ATRA and ATO in combination with DNA vaccine compared to ATO alone or ATRA alone (Figure 64). Similar to the previous results ATRA +DNA gives a significantly prolonged survival than ATO+DNA or ATO alone.

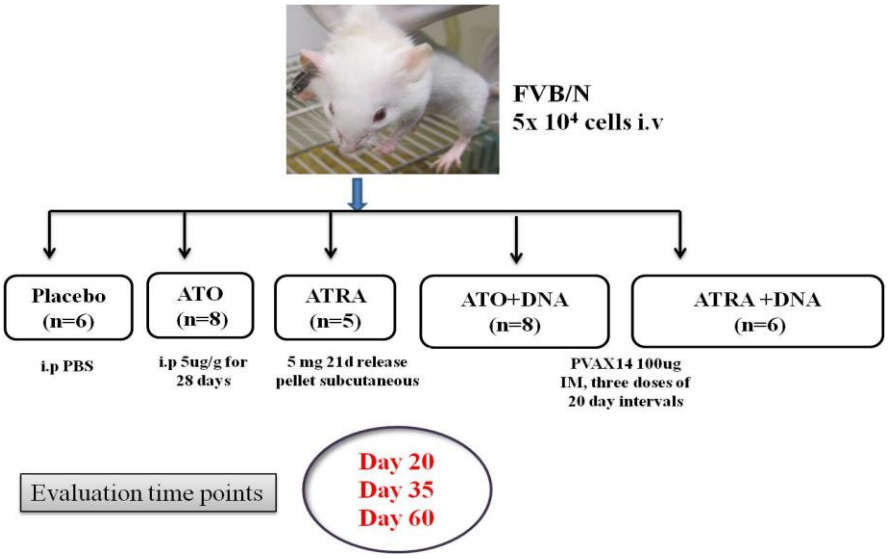


Figure 64: Schematic representation of protocol 2. Treatment of mice with ATO+DNA and ATRA+DNA

WBC and platelet counts

The median WBC count is significantly lower in ATO+DNA group of $2.2x10^3/\mu l$ ($1.2\text{-}12.6\ x10^3/\mu l$) when compared with ATRA +DNA of $9.9x10^3/\mu l$ ($9\text{-}13.64\ x\ 10^3/\mu l$) on Day 35. Whereas in the ATO group alone, the counts increased by day 35 and none of the mice survived beyond day 48. The below graph shows the WBC counts on Day 20

and Day 35 (Figure 65). The median platelet count of ATO+DNA group was 783 x 10^3/µl (743-846) and 678 x 10^3/µl (476-1269) on Day 20 and Day35 respectively and ATRA +DNA group were 870 x 10^3/µl (544-1074) and 818(585-1029) and 745 x 10^3/µl (654-878) on day 20, day35 and day60 respectively.

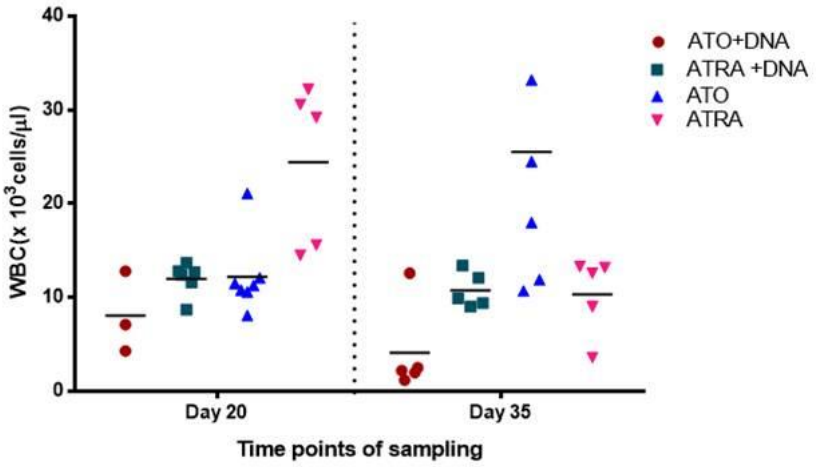


Figure 65: Graph showing the WBC counts of different treatment groups at Day 20 and Day35.

Evaluation of T-memory cells

The blood samples were collected on Day35 and assessed for the presence of T memory cells. The gating strategy for Tm ($CD4^+CD44^{high}$ $CD62L^{low}$) is represented in Figure 66. When compared with ATRA alone group (Tm=4.10±0.9% n=5), ATO alone group has a higher percentage of T memory cells(Tm=6.4±0.8% n=6) which was statistically significant (p=0.008) and the addition of DNA vaccine does not make a

significant change in the percentage of Tm. None of the other treatment group combinations appear to significantly alter this population (Figure 67).

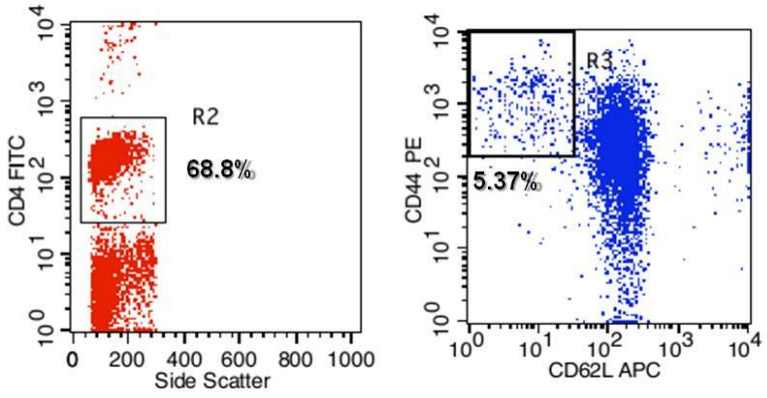


Figure 66. Gating strategy for T memory cells. *From the CD4 gated population* $CD44^{high}$ $CD62L^{low}$ *cells were defined as T memory cells.*

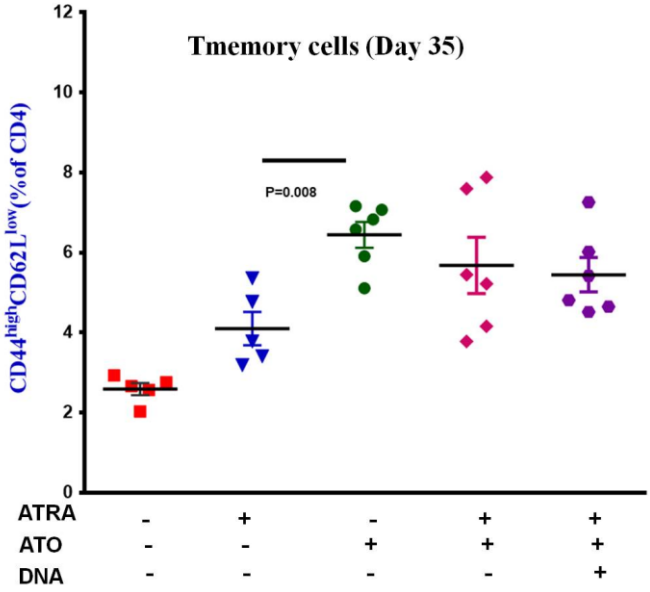


Figure 67. Graph showing the percentage of T memory cells in peripheral blood samples of different treatment groups collected on day35

Survival analysis

Compared with the ATO alone treated group addition of DNA vaccine does not not improve the overall survival analysis with a median survival of 48 days. Whereas the survival of ATRA+DNA group is similar to ATO+ATRA+DNA or ATO+ATRA groups. Figure 68 shows the Kaplan Meier survival curve of different study groups.

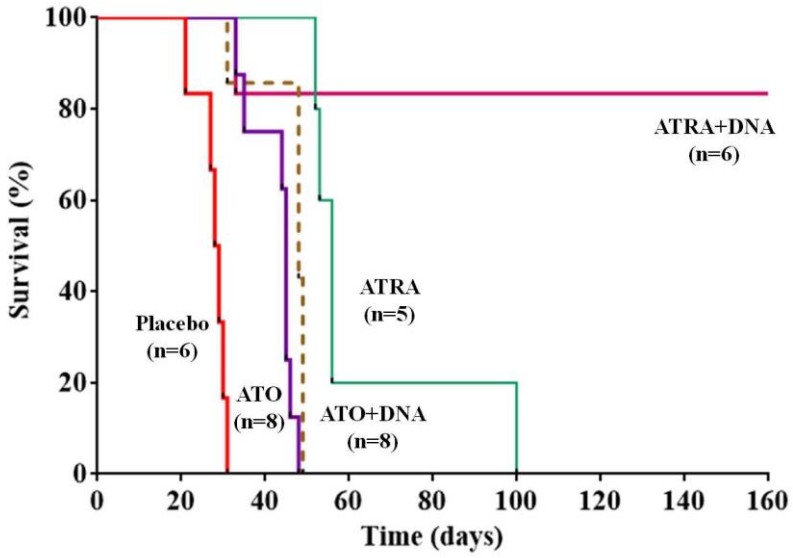


Figure 68: Kaplan Meier survival curves of APL FVB/N treated with ATO, ATRA, ATO+DNA and ATRA+DNA vaccine

4.9.3. Experiments on long-term survivors

Challenging of long-term survivors with APL blasts

Challenge experiments were done on long-term survivors (LTS) to elucidate the role of immune system in clearing up the leukemic cells. The LTS were challenged with $1x10^4$ APL blast cells to the following groups: a) ATO+ATRA (n=6) b) ATO+ATRA+DNA (n=2) c) ATRA+DNA (n=3), d) ATRA (n=1) and e) placebo (n=3) and looked for the survival. The mice were followed up for 60 days and we observed 100% survival in all the groups without any leukemia-related death.

Immunoblotting for confirming the presence of anti RARα antibodies

The specificity of the anti-RARα antibody response was confirmed by western blots using recombinant GST or GST-RARα proteins with either anti-RARα antibody or serum from day 60 of mice treated with ATO+ATRA+DNA vaccine showing the presence of RARα antibodies in the serum (Figure 69).

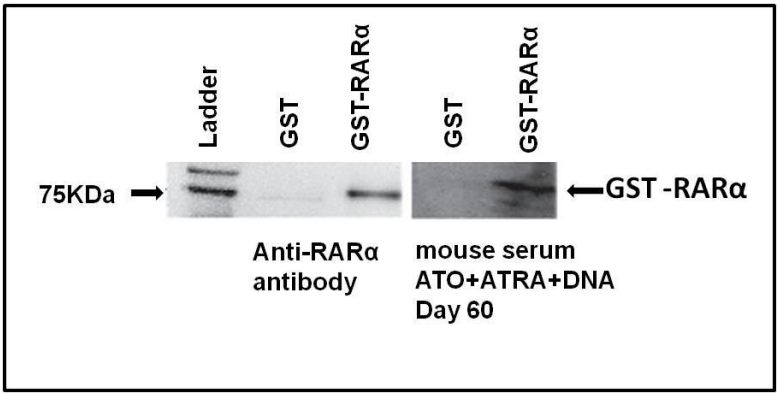


Figure 69. Representative immunoblot showing recombinant GST or GST-RARα proteins probed with serum from day 60 mice treated with ATO+ATRA+DNA *(Santa Cruz, clone C20) (GST -25 kDa and GST-RARα- 75KDa)*

Protocol 3

4.9.4. NK cell therapy in APL mouse model

Since we have observed the role of ATO in activating the NK receptors and ligands in cell lines and patients, we tried to look at the effect of NK cells in survival in our APL mouse model. The sorted NK cells ($CD49b^{+}CD3^{-}$) from the spleen of wild-type FVB/N cells were injected intravenously at a concentration $5x10^{5}$ NK cells per 200µl in 3 doses (Day 8, Day 18 and Day28). We were able to get 11 $x10^{6}$ NK cells post sorting starting with 55 $x10^{6}$ splenic cells. The negative fraction (splenocytes) were injected for the control group. The below dot plots show the percentage of NK in spleen before sorting and the enriched NK population after sorting (Figure 70).

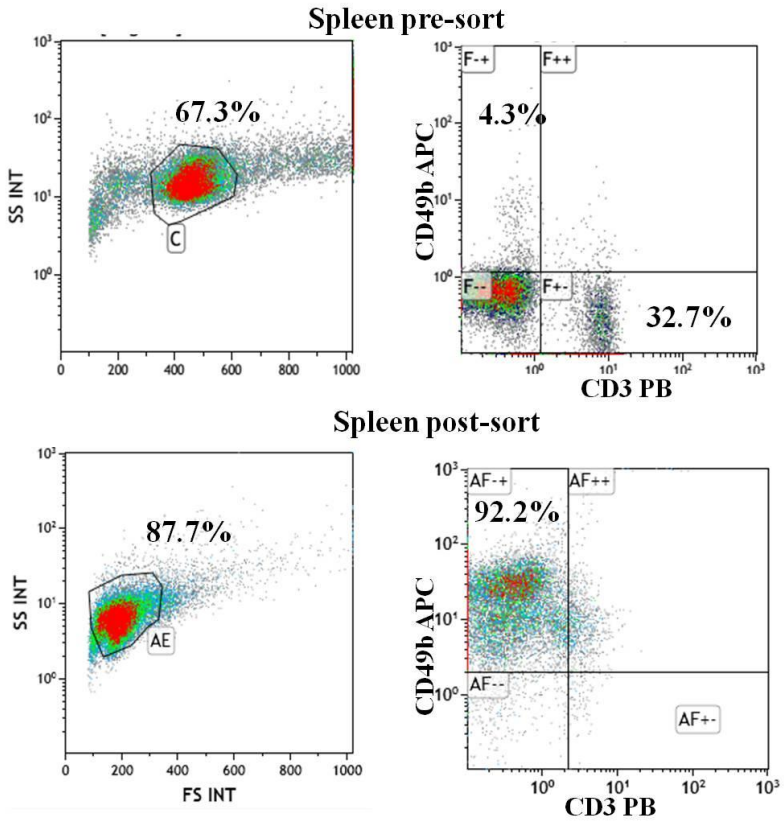


Figure 70: Representative dot plots showing the percentage of NK cells ($CD49b^{+}CD3^{-}$) in spleen pre and post sort. The percentage of NK cells post sorting was 92.2%.

Study design

The mice were separated into five experimental groups: Placebo (n=6), Placebo+NK (n=6), ATO (n=6), ATO+NK (n=6) and ATO+splenocytes (n=6) and the mice were followed up for survival. The group injected with splenocytes served as the control group. NK cells were given in 3 doses with 10 days interval. Figure 71 shows the schematic representation of different arms in this experiment and the interventions done.

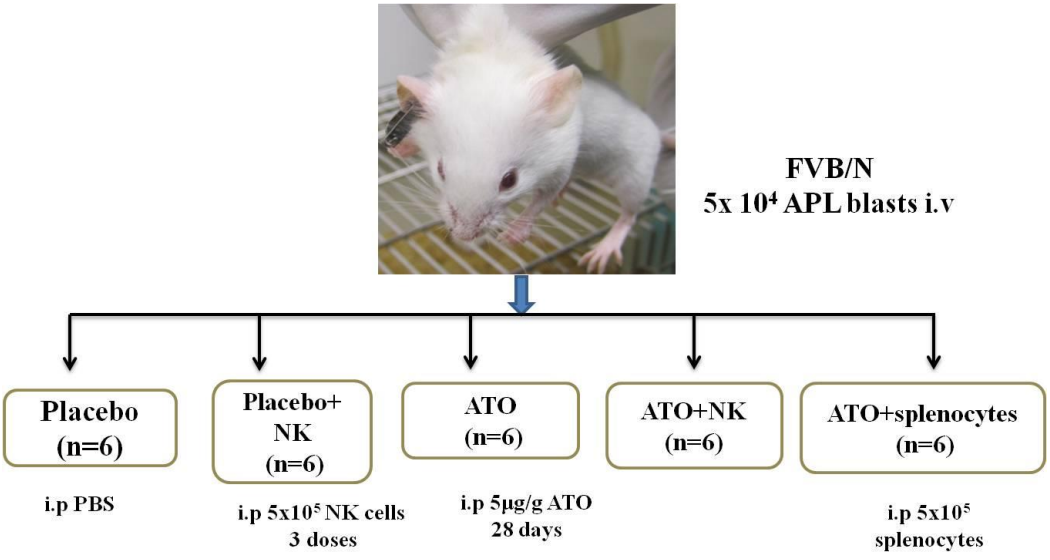


Figure 71: Schematic representation of various treatment groups with NK therapy in APL mouse model.

Survival curve

Compared with the ATO group mice treated with only NK cells did not show any improvement in survival whereas mice treated with ATO along with 3 doses of NK shows a significantly increased survival with a median survival of 54 days (52-75days) when compared with ATO alone with a median survival of 44 days (33-46 days) (p=0.000) (Figure 72).

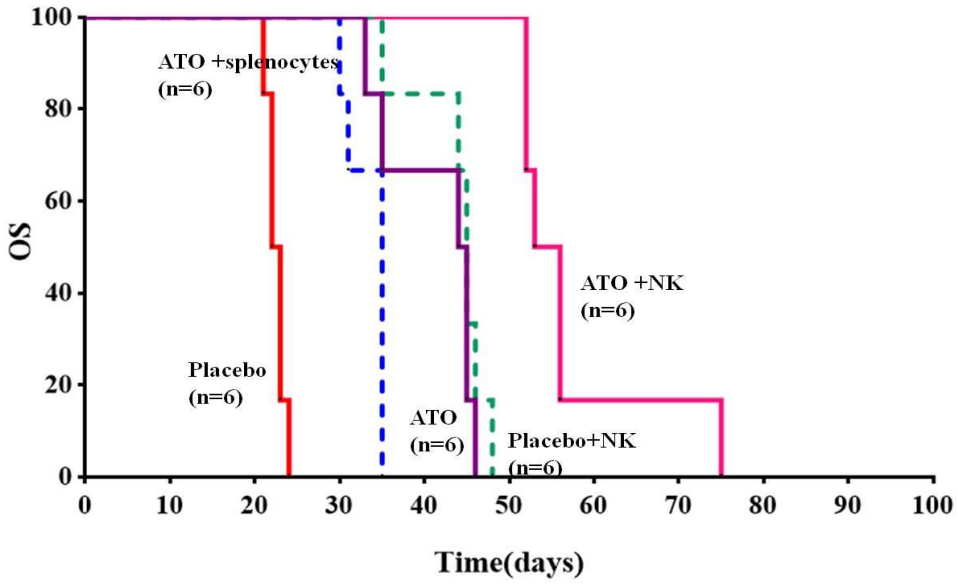


Figure 72: ***Kaplan Meier survival curves of APL FVB/N treated with ATO, NK cells, ATO+NK and ATO+splenocytes.***

Protocol 4

4.9.5. NK cell therapy in APL mouse model with IL-15

We looked at the survival of mice with the addition of IL-15 along with ATO and NK cells. The experimental groups added to the previous were leukemic FVB/N+ATO + IL-15 (n=6) and leukemic FVB/N +ATO + NK+ IL-15 (n=6). The addition of IL-15 along with NK cells had an added advantage in survival over the group treated with ATO alone (Figure 73).

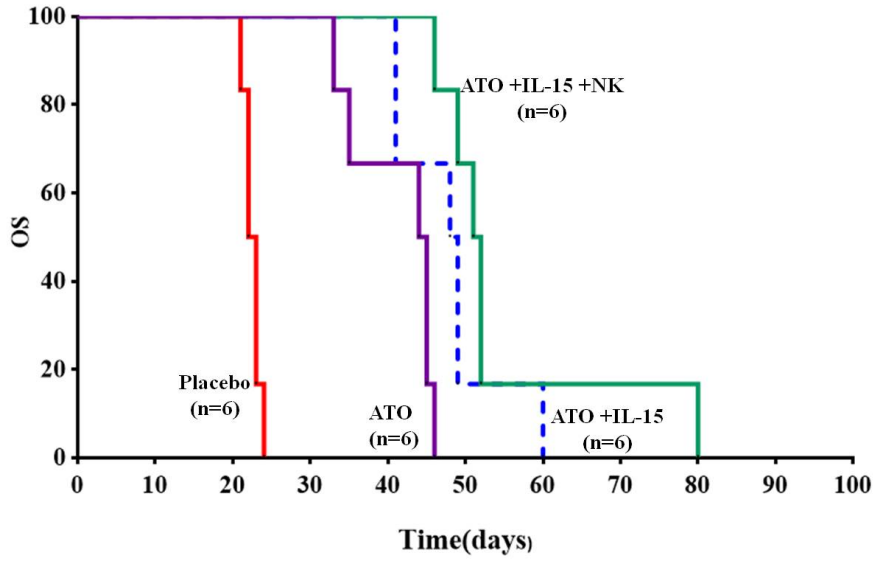


Figure 73: ***Kaplan Meier survival curves of APL FVB/N treated with ATO, ATO+IL-15, and ATO+IL-15+NK.***

DISCUSSION

Our understanding of the human immune system is rapidly evolving. The role of immune system in the control and eradication of cancer has been studied for a long time but the mechanisms have not been studied extensively. The molecular interplays between the immune system and tumor and how the altered immunity drives malignant development and progression is the topic of interest in recent years. During the past two decades we have experienced the shift of methods in cancer treatment from using non-specific cytotoxic agents to selective molecular mechanism-based therapeutics. There are accumulating evidence of anti-cancer chemotherapy in augmentation of host immune reactivity (Shurin et al., 2012). In the field of immunology, there is increased relevance of manipulating the immune response by a vast array of therapeutic agents including monoclonal antibodies, bispecific antibodies, cytokines, adoptive cell transfer, use of check point inhibitors (CTLA-4 and PD-1inhibitors), epigenetic modulators and DC-based vaccines in hematological and non-hematological malignancies. The goal of these therapies is to harness the power of immune cells for a better clinical outcome. Likewise, immunological reconstitution and restoration of functional immune effector cells after an intensive chemotherapy is of prime importance which has not been explored in detail.

In the context of APL, earlier publications from our center showed the efficacy of single agent ATO in terms of durable remissions and minimal toxicity for the treatment of newly diagnosed patients. Interventions to increase the effectiveness of this therapy is our major interest and one aspect of it is through immune modulation.

Although there are data available with ATRA in enhancing adaptive immune response there is limited data on the impact of ATO on immune response and function. Also there is no data comprehensively evaluating the pattern and long-term recovery of the innate and adaptive immune system in APL patients treated with ATO. In this study we tried to understand the effect of ATO in the reconstitution of immune subsets and role of ATO, if any, in modulating the immune responses. To the best of our knowledge, this has not been studied in APL so far. We have also studied the effect of DNA plasmid vaccine in a mouse of APL treated with ATO and the role of NK cell therapy in extending the survival of mice along with ATO.

We had the unique opportunity to work with patient samples treated with single agent ATO and hence this study is unique in terms of studying the effect of ATO on immune reconstitution, recovery and function post treatment. Peripheral blood samples were studied prior to ATO and post ATO therapy for a period of two years to prospectively evaluate cellular reconstitution pattern. In this study cohort, the estimated event-free survival and overall survival at 3 years were 66.7± 5.2% and 91.4± 2.9% respectively. The relapse-free survival (RFS) and RT-PCR positivity at end induction were taken as clinical outcomes for analysis.

On a univariate analysis of patient characteristics with RFS, a higher median WBC count at diagnosis was significantly associated with relapse (p=0.055). Those patients who were RT-PCR positive at end induction also had a higher incidence of relapse with a hazard ratio of 2.6 (p=0.029). Similar results were obtained from previously published data from our group with this regimen (Chendamarai et al., 2012). Other parameters like age, sex, hemoglobin, platelet count, creatinine, coagulation parameters and type of bcr transcripts (given in Table 8) does not had any impact on RFS. In those patients who were RTPCR positive at end induction, the median WBC counts were significantly lower when compared with RT-PCR negative group (p=0.047).

In addition to some of the conventional parameters that could predict the relapse, we hypothesized that the recovery kinetics of immune subsets would also impact the clinical outcomes and predict relapse in APL patients treated with single agent ATO. We also postulated that ATO could modulate the immune responses against APL cells. This doctoral work aimed at evaluating the different aspects of ATO based therapy in APL.

'We are much better at taking cells apart than putting them together. Reconstitution of biological and cellular processes is a powerful yet difficult approach to study'- Allen P. Liu and Daniel A. Fletcher, Nature Reviews, September 2009.

5.1 Differential immune reconstitution pattern in APL

5.1.1. Immune recovery pattern of T cells, B cells and NK cells post ATO therapy

In the context of transplantation, early recovery and reconstitution of innate immunity (granulocytes, monocytes/macrophages and natural killer cells) offer protection against many bacterial pathogens, but the levels of functional lymphocytes frequently remain abnormal for many months or years. Studies have focused mainly on the immune recovery of patients with hematological malignancies who have undergone autologous or allogeneic transplantation. Such patients undergo a prolonged period of immune dysfunction in the post-transplant period that persists long such as quantitative and qualitative abnormalities in B and T cell populations (Guillaume et al., 1998). In leukemia, chemotherapy will have detrimental off target effects on the immune system. However, the patterns of recovery of immune subsets post chemotherapy have never been studied extensively unlike in a post-HSCT setting. Also the impact of the recovery pattern on clinical outcomes was not evaluated in detail. There is no data on the effect of ATO on immune subset recovery post treatment in APL.

In our study, we observed that all the subsets evaluated were below the normal ranges at diagnosis except for dendritic cells, and NKT cells were in the near normal ranges. Also, our study demonstrates that there is a heterogeneity in the pattern of immune reconstitution in different lymphocyte subsets post treatment of APL with ATO. The CD3 T cell subset recovered within 3 months after the initiation of ATO therapy. CD4 T helper cells recovered a little later when compared to CD8 T cytotoxic cells. This resulted in the inversion of the CD4/CD8 ratio which persisted until the start of consolidation therapy even though not statistically significant between the time points. This is similar to our data in a post-transplant setting (Rajasekar et al., 2009). Other studies in post-transplant patients showed that circulating CD3+ T cells recovers shortly (within three months) and patients experience prolonged inversion of the CD4/CD8 ratio due to the relative absence of T helper cells and the increased presence of CD8+ suppressor cells (Olsen et al., 1988). In a study looking at the immune reconstitution in childhood ALL, naive T-cell subsets were more reduced than memory subsets at six months post treatment (Ek et al., 2005). Our study has shown that both the memory T cells and the naïve T cells recovered before the start of consolidation. Similar results were shown in a study by van Tilburg et al. were naive T cell populations were restored within six months in children treated for hematological malignancies (van Tilburg et al., 2011) whereas memory T cells recovered much slower. In contrast, another study in breast cancer patients, memory CD4+T cell post chemotherapy recovered faster than naive CD4+Tcell recovery, and indeed CD4CD45RA cells reconstitute to normal ranges

after two years of therapy (Hakim et al., 1997). When these subsets are correlated clinically with the outcome, a lower median absolute count of CD4CD45RA naïve T cells post-induction therapy (at the time of documenting clinical remission) were significantly associated with relapse (p=0.038). This data has never been reported previously and highlights the potential importance of immune recovery in maintaining durable remissions.

$CD4^{+}CD25^{+}$ activated T cells play a significant role in immune suppression and studies have shown that this subset was significantly higher in ALL and AML patients at diagnosis than in healthy individuals and was associated with poor prognosis (Wang et al., 2005, Shenghui et al., 2011, Wu et al., 2012). In our study CD4+CD25+T cells have recovered to normal ranges by the end of induction and were maintained throughout the therapy. However, the median absolute counts of this subset at diagnosis and at pre-consolidation time point were significantly higher in those patients who relapsed when compared to non-relapsed group (p=0.035 and p=0.050 respectively). Studies on the reconstitution of the B cell compartment after cessation of chemotherapy in pediatric ALL showed a fast recovery of immunoglobulin levels and B cell numbers recovered within months to normal levels (Alanko et al., 1992). In our study, B cells have recovered to normal levels by six months after the initiation of ATO and were in normal ranges until the end of therapy.

NK cells were the first subset to reconstitute post transplant and studies from our center has shown the effect of low day 28 NK count on the risk of graft rejection in allo

BMT (Rajasekar et al., 2009). A study from University Children's Hospital, Tuebingen, Germany showed early recovery of CD56+CD16+ cells by day 14 post-transplant with high cytolytic activity against K562 and strong ADCC activity against neuroblastoma and leukemia blasts (Pfeiffer et al., 2010). Few studies in pediatric B-ALL showed that NK cells remained subnormal during therapy but recovered rapidly after the end of chemotherapy (Alanko et al., 1995, Eyrich et al., 2009). Surprisingly in our study we observed a delay in the recovery of NK and the median NK cell absolute counts were below the normal ranges even a year after initiation of therapy. Different findings in AML patients in CR shows that absolute numbers of CD56+CD16+ cells remains high and gradually decreased to normal levels by two years post therapy (Ohnishi et al., 1998) showing the immune surveillance of NK cells post chemotherapy. It has been demonstrated in previous studies that NK cell activity in the peripheral blood of untreated acute leukemia patients was significantly decreased and an impaired NK activity in early remission and a gradual recovery at a later stage of remission were observed in the context of AML (Talpaz et al., 1982). Our studies in APL patients also showed a delay in the reconstitution pattern of NK subset even a year after the initiation of ATO.

5.1.2. Immune recovery pattern of NK cell subsets

The two major phenotypically and functionally distinct subsets of NK are CD56bright ($CD56^{bri}CD16^{+}$ and $CD56^{bri}CD16^{-}$) which is the immature population and CD56dim subset ($CD56^{dim}CD16^{+}$ and $CD56^{dim}CD16^{-}$) which is the terminal

differentiated cells responsible for cytolytic activity. $CD56^{dim}CD16^{-}$ and the other subset $CD56^{-}CD16^{+}$ which is a dysfunctional subset are numerically in minority. The heterogeneity of NK populations is of great interest in transplant settings. It has been shown that there is an expansion of the cytokine-producing CD56bright cells post-HSCT which could be associated with a cytokine-driven mechanism (Dulphy et al., 2008). Also there are several reports of an increased ratio of CD56bright to CD56dim NK cells at sites of chronic inflammation (Dalbeth et al., 2004). Our analysis has shown that among the CD56 subsets, the median absolute counts of CD56 dim populations were lower and recovered slowly than the CD56 bright population in our cohort. Since CD56 bright are the immediate precursors of the CD56 dim subset, we think there is a delay in the differentiation of CD56bright to CD56dim population probably mediated by the exposure with ATO. Similar results were shown by Carrega et al. describing that tumor-infiltrating NK cells display an immature phenotype (Carrega et al., 2008). When we examined the impact of this NK maturation pattern on clinical outcomes, we observed that the absolute counts of $CD56^{bri}CD16^{-}$ subset were significantly lower in patients those who are RT-PCR positive at the end of induction when compared with RT-PCR negative groups ($p=0.054$) whereas CD56dim population does not show any significant difference in the two groups.

The $CD56^{-}CD16^{+}$ NK subset is shown to be greatly expanded in HIV-viremic individuals and it impairs the NK cytolytic activity (Mavilio et al., 2005). In our patients, this subset lies in the normal ranges till the end of therapy but the median

absolute counts when analyzed at the 2nd cycle of maintenance were significantly higher in relapse patients when compared with non-relapse group (p=0.013). The impact of this subset on clinical outcome in leukemia has not been addressed before in any other studies.

NKT cells are a distinct subset of T cells that share the NK characteristics and rapidly produce an array of cytokines upon activation. NKT cells exert antitumor immunity and are thought to prevent GVHD after bone marrow transplantation. Studies have shown the association of a low number of NKT cells with poor prognosis in AML (Najera Chuc et al., 2012). In contrast, another study showed increased absolute numbers of $CD3^{+}CD56^{+}$ cells in AML patients but was not correlated with increased incidence of relapse (Le Dieu et al., 2009). In our study, we observed that the NKT cells lie in the normal ranges from end induction till the end of therapy and there was no significant association of this subset with the clinical outcome in our study.

In the context of delay in recovery of mature NK subsets, one possibility is that the leukemic cells may impair the NK developmental process from HSC as shown in a recent study stating that IL-1β released by AML blasts inhibit NK differentiation and impaired NK cell recovery (Vitale et al., 2015). However in our study delay on NK cell recovery was noted even after achieving remission including molecular remission. Our data suggests the effect we were seeing is more likely to be induced by ATO rather than the effect of leukemic cells.

5.1.3. Reconstitution pattern of dendritic cell subsets

Dendritic cells are innate immune cells involved in regulation of adaptive immune system and they have inherent tolerogenic properties. Studies from our center showed a low day 28 PC associated with risk for developing aGVHD (Rajasekar et al., 2008) and high PCDC is correlated with improved OS (Mohty et al., 2005). However, studies on reconstitution of DC subsets and their significance were not well explored in leukemia. One study showed increased frequencies of myeloid and plasmacytoid dendritic cells in peripheral blood of AML at diagnosis with *FLT3* internal tandem duplication (Rickmann et al., 2011). In children with B-lineage ALL, numbers of both monocytoid DC and plasmacytoid DC were significantly reduced in peripheral blood at presentation whereas T-lineage ALL patients showed significantly elevated counts of MCDC and slightly elevated PCDC at diagnosis (Maecker et al., 2006). In our patient cohort, we found that both MCDC and PCDC were in the normal ranges throughout the therapy and a higher median absolute count of MCDC at diagnosis was significantly associated with relapse ($p=0.001$). Since DCs have the ability to induce regulatory T cells which suppress the immune response we could correlate with the increased CD4+CD25+T cells and MCDC in relapse patients even though the mechanism is not clear.

5.2. Phenotypic heterogeneity of MDSC subsets in leukemia

The heterogeneous immature myeloid cells MDSCs are present in low frequency in healthy individuals and are increased in inflammation and cancer. The functional relevance was predominantly studied in solid tumors, but limited data is available regarding its presence and role in leukemia. Increased frequency of $CD14^{+}HLA\text{-}DR^{low}$ cells was observed in CLL, and such patients had a shorter time to disease progression (Jitschin et al., 2014). A recent study from Sun et al. showed increased $CD33^{high}CD11b^{+}$ $HLADR^{low}$ cells in AML patients (n=27) when compared to controls but there was no significant relationship between MDSC levels with age, sex and WBC counts (Sun et al., 2015). In our study cohort of AML, ALL and APL, there is a significant heterogeneity between them at diagnosis. The percentage MDSC was low in ALL and APL when compared with AML. Among AML (n=137), the MDSC ranges from 0.01% to 31.1% and 56% of patients have median values above the median of HC. This was in contrast to a study showing that MDSCs in AML-M3 were higher than that in M2, M4 and M5 (Sun et al., 2015).

In our study, there was a significant positive correlation of MDSC in AML patients with WBC and negative correlation with %CD34. Also AML patients in the high quartile of MDSC (>3.74%) was associated with high-risk NPM1+FLT3+. This observation is comparable to the above mentioned study by Sun et al. showing increased MDSC in high MRD group (MRD $>1\times10^{-2}$). Also, the expression level of WT-1 detection level was greater in the high MDSC group. In our study we haven't analyzed

monocytic MDSCs (CD14+CD11b+) and polymorphonuclear MDSCs (CD33+CD11b+) separately. Since this population is originated from myeloid progenitors and immature mononuclear cells, we have to investigate further whether this population is derived from some type of AML clones.

5.3. Presence of anti-RARα antibodies in APL patients and APL mouse model

It has been recognized that, in mice and patients with APL, antibodies against RARα and myeloid proteins are frequently present and potentially contribute to the maintenance of sustained molecular remission (Robin et al., 2006, Patel et al., 2015, Le Pogam et al., 2015). We had evaluated for the first time the presence of anti-RARα antibodies in the serum of APL patients treated with single agent ATO by ELISA. Even though the specific absorbance was higher in patients when compared with the controls, we did not observe any significant increase in anti-RARα antibodies post treatment with ATO. We also showed the presence of anti-RARα antibodies in the long-term follow-up patients (1-year post treatment) and mice (Day 60 APL mice treated with ATO+ATRA+DNA) by immunoblotting. So far no data was reported showing the presence of anti-RARα antibodies in patients treated with ATO. However, we could not demonstrate any significant impact or correlation between anti-RARα antibody response and clinical outcomes which is in contrast with the earlier reports with ATRA.

5.4. Immunomodulatory effect of ATO on NK cells

Recent evidence suggests that immune-mediated mechanisms can enhance the efficacy of the chemotherapeutic agents. Since we observed a delay in the recovery of NK cells in patients treated with ATO and hence we looked at the effect of ATO on NK cells *in vitro*. For all the *in vitro* experiments, ATO was used at a concentration which was not cytotoxic to the leukemic cell lines and the NK cell line used in this study. *In vitro* ATO sensitivity was done on all cell lines by MTT assay.

NK92 MI is a highly cytotoxic natural killer (NK) tumor cell line which has the characteristics of natural killer cell and is IL2 independent. This cell line is cytotoxic to a wide range of malignant cells like K562 and is used for adoptive immunotherapy (Tam et al., 1999). The cell line is characterized phenotypically using an array of markers including the activating and inhibitory receptors. We showed that ATO has no direct cytotoxic effect on this NK cell line (IC50=3.8μM) and there is no change in the rate of proliferation of NK cell line on treatment with 1μM ATO at 24hrs, 48 hrs and 72 hrs.

5.4.1. Cytolytic activity of NK92MI cell line

Natural killer cells appear to play a crucial role in recognition and eradication of leukemic cells. In a study with AML patients receiving haplotype-mismatched stem cell transplants, the capacity of NK cells to kill leukemic cells has been demonstrated (Giebel et al., 2003, Ruggeri et al., 2002). In this study we used NK92MI cell line which

is known to have the antitumor activity and can kill K562 and Daudi (B lymphoblast cell line) cells effectively at the ratio of E:T of 5:1 (Gong et al., 1994). We checked the cytolytic activity against all the myeloid and lymphoid cell lines used in this study by a CFSE/7AAD cytotoxicity assay. We observed that the mean cytolytic activity of NB4 was 21.5±3.7% at the highest E: T ratio of 10:1 tested. As reported previously significant cytolytic activity was noted against K562. The other cell lines do not show a similar kind of cytolytic activity. The least cytolytic activity was noted against the APL resistant cell line NB4EVAsR1. We hypothesized that the cytolytic activity is mediated by the presence or absence of activating and inhibitory ligands on the leukemic cell lines and activating and inhibitory receptors on NK cell line and this could be potentially modulated by ATO. The balance between these receptors decides on whether the target should be lysed or not based on the missing self-hypothesis and NK tolerance mechanisms.

One of the mechanisms of leukemic cells to avoid immune recognition is to downregulate NKG2D and NCR ligands expression. A study has shown the heterogenous expression of NKG2DL in various AML, ALL, breast cancer and colon cancer cell lines and low levels of ligands on AML cell lines correlated with the hypermethylation of NKG2DL (Baragano Raneros et al., 2015). So we looked at the expression of NK ligands MICA/B (ligand for NKG2D), HLA-ABC and CD112 (ligand for coreceptor DNAM1) in all the myeloid and lymphoid leukemic cell lines used in this study. The leukemic cell lines NB4, NB4EVAsR1, U937, K562, Jurkat and SUP B-15

showed a low basal surface expression of MICA/B activating ligand and was negligible in UF1 and HL60. The surface expression of HLA-ABC was completely absent in K562, U937 and NB4EVAsR1. In NB4, even though the HLA Class 1 ligands are present, the increased presence of activating ligands like MICA and MICB drives the NK cell for activation. K562 is a known target of NK which is an HLA class I-deficient myeloid leukemia cell line lysed by the missing self-recognition. The low expression of activating ligands must be one of the reasons for the reduced cytotoxic activity in other cell lines especially in NB4 EvAsR1 and thus escape from NK-mediated recognition. We have the microarray data showing that the immunoregulatory genes in newly diagnosed and relapse APL patients were differentially regulated. Also the ULBP1 activating ligands, HLA-ABC ligand and the Fas ligands are significantly downregulated in NB4EVAsR1 when compared to NB4 naïve (unpublished data).

A few studies have already shown decreased sensitivity of drug-resistant cells (doxorubicin-resistant leukemia and neuroblastoma cell lines) towards T and NK cell cytotoxicity (Friesen et al., 1997, Classen et al., 1999). Another study had shown a sharp down-regulation of HLA-class I molecules in various leukemia (Pende et al., 2005) and reduction of MICA/B surface expression that may impair NKG2D-mediated immune surveillance of leukemia (Hilpert et al., 2012). Studies in AML showing the presence of AML blasts causing defects in the natural cytotoxicity receptors (NCRs) NKp46 and NKp30 (Costello et al., 2002). It has also been shown that NK cell cytolysis of AML was significantly higher than in tested ALL blasts indicating that activating factors such

as DNAM-1 ligands and NKG2D ligands are involved in the cytolysis of AML blasts (Salih et al., 2003).

5.4.2. ATO was able to enhance the NK receptors expression

Studies have shown that IL-2 activated NK cells have increased lytic activity compared with circulating NK cells and also shown the importance of activating receptors in regulating NK cell effector function (Caligiuri et al., 1990). Also impaired NK cell function and cytokine production is associated with early relapse in adult acute leukemia (Tajima et al., 1996). The activation of NK cells and its recognition of triggering ligands on the surfaces of leukemic blasts confer susceptibility to NK-mediated lysis. From our observation, NK cell mediated cytotoxicity was prominent against malignant myeloid cell lines which were not seen or less prominent against lymphoid cell lines. The NK cell-mediated cytotoxic effect was seen prominently against K562 and NB4 cell line. These observations lead us to evaluate mechanism to explain this effect.

We investigated the NK receptor repertoire and evaluated the effects of ATO and we demonstrated that the receptors on NK92MI cell line were upregulated in the presence of ATO. On exposure of NK cell line with 1μM ATO for 6 hours, we observed an increased expression of activating receptors NKG2D, Nkp30 and KIR2DS4 and inhibitory receptor NKG2A. Downregulation of inhibitory receptor KIR3DL1/DL2 was

also observed. Our data suggests that the concentrations at which ATO was used gives an immunomodulatory effect and activates innate immunity.

5.4.3. Expression of NK ligands induced by ATO

We then evaluated the effect of ATO in altering the NK ligand expression on leukemic cell lines. Except for NB4 none of the other cell lines showed a significant increase in expression of NK ligands on exposure to ATO. The NKG2D ligands expressed in promyelocytic cell lines was significantly increased upon priming with ATO when compared with the other myeloid and lymphoid cell line tested and. Similar observation was seen in one other study showing upregulation of NKG2D ligands, MICA/B and ULBPs by ATO in K562, NB4 and MCF7 breast cancer cell lines and increased susceptibility to NK-mediated cytotoxicity. In K562 the cell surface expression of MICA/B remains unaltered with ATO treatment (Kim et al., 2008). Valproic acid (HDAC inhibitor) and ATRA was also shown to promote NKG2D ligand expression on several monocytic and lymphoid cell lines and in primary AML cells suggesting that these drugs might sensitize tumor cells to immune cell killing (Poggi et al., 2009). The chemotherapeutic agents like anthracyclines, cyclophosphamide and oxaliplatin also regulate NK cell recruitment and activity but may be dependent on cancer type or dosing strategy (Brenner and Margolese, 1991). Thus ATO seemed to have immunomodulatory effects besides its role in the degradation of PML-RARα.

5.4.4. Increased cytolytic activity of NK cells by ATO

Our data suggests that pretreatment of leukemic cells or NK cell line with ATO alters the cytolytic effect. We observed that exposure of NB4 cell line to 1μM ATO for overnight significantly increased the cytolytic activity when compared to the untreated. All the other cell lines tested could not increase the cytotoxic effect of NK as significantly as NB4 cell line. The increased NK cytotoxicity after ATO treatment correlates with the increase in expression levels of MICA, MICB and DNAM-1 on NB4 cell line. Thus modulation of the expression of the NK-cell receptors and ligands by ATO increased NK cell-mediated cytotoxicity and thus could potentially exploit the NK cell anti-tumor effects. Studies have already looked at the effect of various immunomodulatory drugs like lenalidomide on CLL showing enhanced ADCC activity against primed CLL cells with NK(Ramsay and Gribben, 2009).

The increased cytolytic activity was also confirmed by CD107a degranulation assay. Even though the HLA class I-deficient myeloid leukemia cell line K562, is a prototype NK cell target, treatment with ATO did not increase the cytotoxicity of NK as measured by the percentage of CD107a. Whereas in NB4 stimulation with 1μM ATO significantly upregulated CD107a on the surface of NK cells (33.6±3.8%) showing increased NK cell-mediated lysis of target cells when compared to untreated (20.4 ±1.8%). It is known that CD107a is upregulated on NK cells following stimulation (Alter et al., 2004).Thus we demonstrated that exposure of APL cells with ATO was able to stimulate the NK cells towards increased cytolytic activity which was not seen in

any other lymphoid or myeloid cell lines tested. The important thing to be noted that we have used only sub-lethal doses of ATO for all the experiments which do not kill any of the cell lines.

5.5. Genotype and haplotype analysis of KIR genes

Natural killer cell alloreactivity, determined by donor Killer-cell immunoglobulin-like receptors (KIRs) and recipient HLA, correlates with successful HCT for AML. Previous HCT studies have reported varied and sometimes inconsistent associations between KIR haplotype and clinical outcome. It has also been shown that KIR immunogenetics play a major role in shaping the immune response to leukemia. Donors with group B KIR haplotypes improved the clinical outcome in unrelated hematopoietic cell transplantation in AML (Cooley et al., 2009). The presence of KIR2DS1, an NK activating receptor, significantly reduced the risk of relapse in AML patients undergone HSCT from matched unrelated donors (Venstrom et al., 2012). It has previously been reported that there is a decrease in KIR2DS3 in AML patients compared to control group (Shahsavar et al., 2010). We have done an extensive study to evaluate the KIR genotype and haplotype in APL patients. Among the 55 patients screened, 10 patients have haplotype A and 45 patients were of haplotype B. The KIR genotype and haplotype on disease susceptibility and whether it could predict relapse has also been investigated in our study. We have not seen any associations with any of the KIR genes to predict either susceptibility or relapse in our cohort. However, we have seen a trend towards an association of KIR2DL2 inhibitory receptor on relapse ($p=0.069$) which is an

HLA-C specific KIR repertoire. One other study also showed the increased frequency of KIR2DL2 in AML and ALL (Verheyden et al., 2004). KIR2DL2 inhibitory receptor is also found to be expressed in the early stages of NK cell differentiation from hematopoietic progenitors (Miller and McCullar, 2001).

5.6. Effect of ATO on NK differentiation from CD34

The discrete stages of NK development are revealed from the *in vitro* and *in vivo* NK differentiation studies from hematopoietic precursors (Freud et al., 2006). Since we observed a delay in the recovery of NK subset in patients undergoing treatment with ATO, we moved on to check whether ATO has any role in affecting the NK maturation and differentiation pathways. Sorted CD34+ cells were differentiated to NK with or without the presence of ATO and the cell count and differentiation pattern was observed on day 8 and day14. We noted a reduction in cell numbers in CD34 culture with ATO and decreased $CD56^{+}CD3^{-}$ population inferring that NK differentiation is delayed in the presence of ATO . The other subsets remain unaltered except that there was a slight increase in the myeloid compartment by day 14 of CD34 differentiation on exposure with ATO. One aspect of this NK differentiation defect could be attributed to the effect of ATO on NK transcription factors involved in different stages of NK development and maturation. Additional experiments are required to clarify the mechanism by which ATO appears to affect the differentiation of NK cells.

5.7. Role of transcription factors in NK cell differentiation

Even though the precise hierarchy of transcription factors governing NK cell maturation is incompletely understood there are studies which have looked at the role of TFs in different stages of NK development. Studies in mice models showed a lack in transcription factors Eomesodermin (Eomes) and T-bet failed to develop NK cells (Gordon et al., 2012). The transcription factors ID2, TOX, and E4BP4 (Nfil3) are thought to specify the earliest stages of NK cell development (Aliahmad et al., 2010). Tox is required for the maturation of NK cells and was found to be upregulated during *in vitro* differentiation of NK cells from CD34 cells derived from umbilical cord blood (UCB) (Vong et al., 2014). Also Tox could directly upregulate the transcription of TBX21. Other transcription factors, including Blimp-1, Ets-1, GATA-3, IRF-2, MEF, and PU.1 play specific roles at distinct stages of NK cell development and maturation. Deletion of Eomes from mature NK cells caused reversion to a more immature state. T-bet and Eomes are two molecular determinants that control transit through unique NK cell maturational checkpoints. E4bp4$^{(-/-)}$ mice showed severely reduced numbers of mature NK cells and disruption in NK cell development (Kamizono et al., 2009). Defects in NK cell development has been showing in mice those lacking genes encoding transcription factors Ets1, Id2 and Gata3.

In our study, we observed a significant decrease in the expression of Tox and Ets-1 in day14 CD34 cells treated with ATO when compared to untreated which is required for the NK maturation and differentiation ($p=0.002$). The other TFs like Ikzf2,

Prdm1, and Klf4 were also decreased in ATO-treated cells. In our study, Eomes and Tbx21 were not downregulated on day14 CD34 with ATO.

5.8. NK cell immunotherapy in APL mouse model

Susceptibility of leukemia cells to natural killer cell attack justifies the application of immune strategies to control the residual blast cells. Allogenic NK cell adoptive transfer is used to induce remission in patients with leukemia (Miller et al., 2005). Since there is increased knowledge of the role of NK cells in tumor surveillance, therapies to boost NK immunity has emerged. The use of cytotoxic agents alone results in significant toxicities and frequently acquired resistance and they will have a very narrow therapeutic index. Combining targeted therapies with immunotherapy results in inhibition of molecular pathways of tumor growth along with stimulation of host immune response which is long-lived. Immunotherapy can induce long-lasting responses by generating anti-tumor immune memory.

Translating the observation from this study that ATO enhances the functional properties of NK cells, we thought to study the effect of NK therapy in a mouse model of APL we established in our lab. We hypothesized that infusing NK cells could increase the antileukemic activity and hence prolong the survival of mice treated with ATO. We observed that NK cells when infused along with ATO for three doses extended the survival in APL mice when compared with those treated with ATO alone. Another strategy to improve the activity of NK was the systemic administration of cytokines like

IL-1, IL-12, IL-15, IL-18, IL-21, IFNs, etc. NK cells preactivated with IL-12, IL-15 and IL-18 are shown to induce enhanced functional responses against primary AML blasts (Romee et al., 2016). IL-15 was known to be effective in augmenting NK cell-mediated cytotoxicity. IL 15 was administered at a concentration of 100ng/mouse along with ATO and NK cells for five doses. The addition of IL-15 along with NK therapy had an added advantage on survival even though not statistically significant. This could be a promising approach to enhance NK cell therapy.

5.9. Efficacy of DNA vaccine as an adjuvant to ATO

Vaccines are administered to stimulate or restore the ability of immune system to fight disease. DNA vaccines have been shown to develop strong and persistent cell-mediated and humoral immune responses to the antigen encoded by the plasmid. It has been demonstrated that an adaptive immune response is important in sustaining long-term molecular remission in a transplantable mouse model of APL. It has also been shown, in a mouse model, that PML-RARα targeted DNA vaccine can induce protective immunity and can synergize with ATRA to produce durable remissions (Padua et al., 2003). An adjuvant effect of ATRA on the immune responses may also contribute to the efficacy of this agent in inducing and sustaining molecular remission beyond that of its known cellular effects of differentiation in APL. We have demonstrated the synergistic effect of ATRA, ATO and DNA plasmid vaccine targeting PML-RARα and resulted in significantly extended survival. Similar observation was noted by the French group

which we collaborated on the mouse model work showing the advantage of pVAX14 DNA add-on therapy with ATO and ATRA (Patel et al., 2015). We also demonstrated the absence of synergy with this vaccine with ATO alone.

Despite significant achievements in the management of APL and the elucidation of mechanisms of action of ATO on APL cells, the understanding of immunomodulating pathways and crosstalk of immune system with leukemic cells remain largely unknown. Cancerous cells escape from the immune recognition under a selection pressure created by the immune system. Restoring these immune response mechanisms may be one way of reducing the risk of relapse in patients with leukemia.

In this doctoral work, we have demonstrated for the first time the role of the innate immune system in patients with APL. We noted a delayed recovery of NK cells in patients treated with ATO but also demonstrated *in vitro* up-regulation of NK cell receptors and ligands on malignant promyelocytes in a direction that enhances NK cell-mediated cytolytic activity against malignant promyelocytes. In an APL mouse model, we were able to prolong the survival in ATO-treated mice which were given NK therapy along with IL-15 when compared with ATO alone. These observations again have significant clinical implications and potential for translation into the clinic. We have demonstrated the synergistic effect of ATRA and a DNA plasmid vaccine in the APL mouse model. We also demonstrated the absence of synergy with this vaccine when ATO was used as a single agent. This data has clinical implications on how we would plan protocols to move forward with the use of this DNA plasmid vaccine in the clinic.

We have for the first time demonstrated the presence myeloid-derived suppressor cells (MDSC) in patients with acute leukemia. While not seen in significant numbers in APL there is significant heterogeneity in patients with AML, and the relevance of this population in AML needs further evaluation.

Limitations of the study and future directions

The observations from this study explains that ATO plays an important role in modulating the NK cell anti-tumor activity. This study can be further evaluated using primary autologous NK cells to measure the cytolytic activity against primary blasts upon stimulation with ATO. It is important to study the impact of ATO on the NK receptors and ligands in APL patients. Also, we could check for receptor activation by blocking with neutralizing antibodies specific for the NK cell activating receptors. We have to elucidate further the pathways involved in ligand and receptor activation. Also, the mechanisms of ATO in affecting the NK differentiation have to be studied further in detail. The mechanism to explain the delayed recovery of the NK subset post treatment with ATO needs further evaluation. It would be ideal to study the immunotherapy in the APL mouse model with different doses of NK cells and IL-15 and to observe its effect more closely in enhancing the immune responses. The data obtained from the *in vitro* observations in this study holds promising and will have great implications in the clinic.

SUMMARY AND CONCLUSION

There is increased recognition of the role of an intact and responsive immune system in achieving cure in cancer. Manipulation of immune cells as well as tumor cells by therapeutic agents and their ability to synergize with other drugs helps in reduction of tumor burden as well as to maintain durable remissions. Acute promyelocytic leukemia once fatal has now become the most curable disease due to the use of differentiation agents and targeted therapy. Other than the known mechanisms of ATO and ATRA in the success of curing APL, its role in modulating the immune system is less explored and has not been studied in APL so far. This doctoral study explores and has comprehensively evaluated the mechanisms of ATO in affecting the immune reconstitution in patients treated with single agent ATO and evaluated the role of ATO in driving the innate immune responses. The effect of ATO on antibody response to RARα was also studied. We also analyzed the ability of pVAX14 DNA vaccine in synergizing with ATO on improving the survival in an APL transplantable mouse model. The potential role of immunotherapy by NK cell infusions and IL-15 administration was also studied in the APL mouse model.

Key observations of the study

- *Our study demonstrates that there is heterogeneity in the pattern of immune reconstitution in different lymphocyte subsets post treatment of APL with ATO.*

This is the first study that extensively evaluated immune reconstitution in APL patients treated with single agent ATO prospectively.

- *The delay in the NK reconstitution pattern in APL patients treated with single agent ATO was a novel observation. It is important to note that after induction none of these patients have received any myelotoxic drugs such as anthracyclines or cytosine arabinoside as part of consolidation or maintenance therapy. To the best of our knowledge, this issue has never been addressed earlier. Further studies are required to understand the relevance and significance of this observation.*

- We *have demonstrated in vitro for the first time that there is a significant increase in NK cell-mediated cytolytic activity against malignant promyelocytes exposed to ATO even at relatively low E: T ratios.*

- *Exposure to sub-lethal doses of ATO enhances receptor expression on NK cells.*

- *Exposure to sub-lethal doses of ATO up-regulates NK ligands on malignant promyelocytes. Thus, modulation of ligands using therapeutic agents is an attractive strategy for NK cell-based immunotherapy.*

- *Exposure to ATO enhances NK ligands and receptors in a direction that favors NK cell-mediated cytotoxicity. This could be an important mechanism by which ATO induces durable remissions in patients with APL.*

- *For the first time, KIR haplotype was explored in APL patients though we could not find any associations with KIR genotypes or haplotypes on clinical outcomes. However, we have seen a trend towards an association of KIR2DL2 inhibitory receptor on relapse.*

- *The study has examined some evidence of ATO affecting the NK differentiation by the downregulation of some NK transcription factors involved in NK differentiation and maturation.*

- *In an APL mouse model, infusion of natural killer cells has increased the antileukemic activity of ATO and hence extended their survival when compared to ATO alone group.*

- *These pre-clinical data shed light on the potential future use of for NK cell-based therapies for APL patients which could be further enhanced by ATO.*

- *We also showed the presence of anti-RARα antibodies in long term follow up patients treated with single agent ATO.*

- *The efficacy of pVAX14 DNA vaccine was tested in APL mouse model and we showed that it could extend the survival when given in combination with ATRA and ATO, but we could not find any synergistic effect with ATO alone.*

- *This doctoral work comprehensively evaluated the role of ATO in modulating the immune responses.*

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8. LIST OF PUBLICATIONS

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1. **Ansu Abu Alex**, Hamenth Kumar, Saravanan Ganesan, Nithya Balasundaram, Kavitha M. Lakshmi, Biju George, Aby Abraham, Alok Srivastava, Rose Ann Padua, Christine Chomienne, Poonkuzhali Balasubramaniam, Vikram Mathews. NK cell mediated cytotoxicity against malignant promyelocytes enhanced by arsenic trioxide: Potential clinical relevance, **Best Abstract Award,** 7th International Haematologic malignancies Conference: BTG 2016, Guangzou, China (Oral presentation).

2. **AnsuAbuAlex,**Ezhilarasi Chendamarai**,**Saravanan Ganesan, Nithya Balasundara m**,** Hamenth Kumar Palani, Sachin David and Vikram Mathews. Arsenic Trioxide Resistance: More to It Than Mutations in PML-RARα, Blood (ASH Annual meeting abstracts) 2014,124:3605, American Society of Hematology, December 6-9, 2014, San Francisco, California (poster presentation).

3. **Ansu Abu Alex,** Saravanan Ganesan, Ezhilarasi Chendamarai, Kavitha M. Lakshmi, Poonkuzhali Balasubramanian, , Biju George, Alok Srivastava, Vikram Mathews. Patterns of immune reconstitution in patients with acute promyelocytic leukemia treated with single agent arsenic trioxide and its impact on time to molecular remission, 6th International symposium on Acute Promyelocytic Leukemia , Sep 29-Oct 2, 2013, Rome , Italy (oral presentation)

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5. **Ansu Abu Alex**, Hamenth Kumar P, Saravanan Ganesan, Nithya Balasundaram, , Kavitha M Lakshmi, , Biju George, Aby Abraham, , Auro Viswabandya , Alok Srivastava, Rose Ann Padua, Christine Chomienne, Poonkuzhali Balasubramanian and Vikram Mathews. NK Cell Mediated Cytotoxicity Against Malignant Promyelocytes Enhanced By Arsenic Trioxide: Potential Clinical Relevance. Blood (ASH annual meeting abstracts) 2013. 122:1455, American

Society of Hematology December 07 -10, 2013; New Orleans, USA **(travel grant award for poster**).

9. APPENDICES

A1. CHEMICALS AND REAGENTS

2-propanol	Sigma-Aldrich
7-AAD	BD biosciences
Agarose	Merck
Ampicillin	Cadila Pharmaceuticals
APS	Calbiochem
ATO	Intas Pharmaceuticals
ATRA pellets	Innovative Research of America
B-PER bacterial protein extraction reagent	Thermo Fisher
Bradford reagent	Bio-rad
BSA	Sigma-aldrich
Chloramphenicol	Cadila Pharmaceuticals
Chloroform	Fisher Scientific
CsCl	Sigma-Aldrich
DMEM	Gibco
DMSO	WAK-Chemie Medical GmbH
DNA ladder	Promega
Ethanol	Hayman Ltd
Ethidium bromide	Bio-Rad
FBS	Gibco
Ficoll paque	GE Healthcare
Folic acid	Sigma-Aldrich
Glycerol	Sigma-aldrich
Horse serum	ATCC 30-2040
IL-15	Biolegend
IMDM	GE Healthcare
Inositol	Sigma-Aldrich
IPTG	Sigma-Aldrich
Isopropanol	Sigma-Aldrich
Kanamycin	Lupin laboratories
L-glutamine	Gibco
Lithium Chloride	Sigma-Aldrich
Lysozyme	Sigma-aldrich

Minimal essential media	Stem cell technologies
MGG	Merck
MTT	Biotium
PEG	Sigma-Aldrich
Pen strep	Gibco
RPMI	Gibco
SDS	Sigma-Aldrich
SOC medium	Invitrogen
Sodium azide	Sigma-aldrich
TEMED	Sigma-Aldrich
Tris	Merck
Triton X-100	Sigma-Aldrich
TRIzol	Invitrogen
Tween-20	Sigma-Aldrich
β-Mercaptoethanol	Gibco

Antibodies for immunoblotting

RARA C-20	Santa Cruz
GST B-14	Santa Cruz
Anti mouse IgG	Cell Signalling
Anti rabbit IgG	Cell Signalling

Kits used

Gentra Pure Blood Kit	Qiagen
ACT1 Cytotoxicity Assay kit	Cell Technology
Superscript II Reverse transcriptase	Invitrogen
Taqman gene expression assays	Applied Biosystems
BL21-one shot chemically competent E.coli	Invitrogen
Plasmid Mini prep	Qiagen
GST Spin Purification kit	Pierce
cOmplete Lysis M reagent kit	Roche
KIR typing kit	Miltenyi Biotech
Super signal West Femto Maximum Sensitivity substrate	Pierce
Easy Sep Negative selection Mouse NK Enrichment Kit	Stem cell technologies
Easy Sep Human CD34 positive selection kit	Stem cell technologies

Flow cytometry antibodies

Human antibodies				
Antigen	Flurochrome	Clone	Catalog no	Vendor
IgGI	FITC	X40	349041	BD biosciences
CD3	FITC	UCHT1	555332	BD pharmingen
CD4	FITC	SK3	347413	BD biosciences
CD14	FITC	M5E2	555397	BD pharmingen
Lineage	FITC	-	340546	BD biosciences
CD45	FITC	2D1	347463	BD biosciences
HU CD 226 (DNAM 1)	FITC	DX11	559788	BD pharmingen
HU CD158b(KIR2DL2)	FITC	CH-L	559784	BD pharmingen
HLA-ABC	FITC	G46-2.6	555552	BD pharmingen
CD94 (NKG2A)	FITC	HP-3D9	555888	BD pharmingen
IgGI	PE	X40	349043	BD biosciences
CD19	PE	SJ25C1	340364	BD biosciences
CD8	PE	HIT8a	555635	BD pharmingen
CD16	PE	B73.1	347617	BD biosciences
CD123	PE	9F5	340545	BD biosciences
CD25	PE	2A3	341009	BD biosciences
CD33	PE	P67.6	347787	BD biosciences
CD34	PE	8G12	348057	BD biosciences
CD158a(KIR2DL1)	PE	HP-3E4	556063	BD pharmingen
HU CD335 NKp46	PE	9 E2	557991	BD pharmingen
HU CD336 (NKp44)	PE	P44-8.1	558563	BD pharmingen
HU CD337 (NKp30)	PE	P30-15	558407	BD pharmingen
HU CD112 (Nectin-2)	PE	R2.525	551057	BD pharmingen
MIC A/B	PE	6D4	558352	BD pharmingen
CD158e/k (KIR3DL1/DL2)	PE	5.133	130-095-205	Miltenyi biotech
CD158i(KIR2DS4)	PE	JJC11.6	130-092-680	Miltenyi biotech
CD314(NKG2D)	PE	1D11	557940	BD pharmingen

IgGI	PerCP	X40	349044	BD biosciences
CD3	PerCP	SK7	347344	BD biosciences
HLADR	PerCP	L243	347364	BD biosciences
CD14	PerCP	MϕP9	340585	BD biosciences
IgGI	APC	X40	340442	BD biosciences
CD45RO	APC	UCHL1	340438	BD biosciences
CD45RA	APC	HI100	550855	BD pharmingen
CD56	APC	B159	555518	BD pharmingen
CD11c	APC	B-ly6	559877	BD pharmingen
CD11b	APC	D-12	340937	BD biosciences
CD107a	PE	H4A3	555801	BD pharmingen
Mouse antibodies				
CD4	FITC	GK15	100406	BioLegend
CD49b	APC	DX5	108910	BioLegend
CD3	PB	17A2	100214	BioLegend
CD44	PE	IM7	103023	BioLegend
CD62L	APC	MEL-14	104411	BioLegend
Gr1	APC	RB6-8C5	108412	BioLegend
CD117	PE	2B8	105808	BioLegend
CD45	PerCP	30-F11	103130	BioLegend
CD19	PE	6D5	115508	BioLegend
CD8	PE	53-6.7	100707	BioLegend

Buffers and Media

Flow cytometry

Phosphate Buffer Saline (10X)

NaCl	90.00 g
NaH_2PO4	2.60g
Na_2HPO4(Anhydrous)	11.50g
Distilled water	1000ml
Adjust pH to 7.2-7.4	
Store at 4^oC	

FACS Buffer

1% BSA and 0.1% sodium azide is added to 1x PBS (freshly prepared)

RBC Lysis solution

NH4Cl	80.20g
NaHCO3	8.40g
Disodium EDTA	3.70g
Distilled water	1000ml
Adjust pH to 7.4	
Store at 4^oC	

Agarose gel electrophoresis

10X TBE

TRIS Base	108g
Boric acid	55g
0.5M EDTA	40ml
Distilled water	1000ml

50X TAE

Tris base	24.2 g
Glacial acetic acid	28.55 ml
Distilled water	1000ml

6X loading dye

Bromophenol blue	12.5mg
Glycerol	1.5ml
Water	3.5ml

SDS-PAGE

7.5% Resolving gel

Water	4.8ml
1.5M Tris pH 8.8	2.5ml
30% acrylamide	2.5ml
10%SDS	100µl
10%APS	100µl
TEMED	8ul

4% Stacking gel

Water	2.22ml
0.5M Tris pH 6.8	1ml
30% acrylamide	680µl
10%SDS	40µl
10%APS	40µl
TEMED	4µl

10%SDS

SDS	1g
Water	10 ml

10% APS

APS	0.2 mg
Water	200 µl

SDS PAGE Running Buffer (1X)

Glycine	14.4 g/L
Tris	3.03 g/L
SDS	1 g/L

5X loading buffer

0.5M Tris pH 6.8	6.25ml
Glycerol	5ml
SDS	1g
β-Mercaptoethanol	0.5ml
Bromophenolblue	0.25g

Coomassive Brilliant blue staining

50% Methanol
10% glacial acetic acid
0.1g CBBR-250

Destaining solution

40% Methanol
10% glacial acetic acid

Western Blotting

Western Blot transfer Buffer (1X)

Glycine	14.4 g/L
Tris	3.03 g/L
Methanol	200 mL

10X TBS and 1X TBST buffer

10X TBS

Tris base	24.2g
NaCl	80.0g

pH 7.6

1X TBST

10X TBS	50 ml
Water	450 ml
Tween20	250 µl

1XPBST

10X PBS	50 ml
Water	450 ml
Tween20	250µl

Blocking reagent

Non fat dry milk 2g in 20ml TBST

Ampicillin-stock

Ampicillin	10g
Water	100ml

Luria Bertani media and agar

LB Powder	3g
Water	100ml

For ampicillin plates 100 µl of ampicillin was added to 100ml of luke warm LB agar before pouring.

Solutions for ELISA

1. Bicarbonate Buffer 0.05mM pH 9.6: One bicarbonate capsule dissolved in 100ml of distilled water
2. Blocking solution: 500mg BSA in 10ml 1xPBS
3. Dilution buffer for Primary Antibody: 1x PBS with 0.5% BSA and 0.05% tween-20.Then 1/10 dilution of the above with washing solution (store at 4°C).
4. Dilution buffer for Secondary Antibody: 1x PBS with 0.1% BSA and 0.01% tween-20.
5. Washing solution: 1xPBS with 0.05% tween-20
6. 1M H_2SO_4

A2. CONSENT FORM

Dr. Vikram Mathews, PI

DEPARTMENT OF HAEMATOLOGY, CHRISTIAN MEDICAL COLLEGE, VELLORE

INFORMED CONSENT FORM FOR SAMPLE COLLECTION EVALUATION OF CELLULAR AND IMMUNE RESPONSE IN MICE AND PATIENTS WITH ACUTE PROMYELOCYTIC LEUKEMIA TREATED WITH ARSENIC TRIOXIDE

I have been informed that scientists and physicians at the Department of Haematology, Christian Medical College, Vellore are engaged in the studies on Evaluation of cellular and immune response in mice and patients with acute promyelocytic leukemia treated with arsenic trioxide.

I have been informed that blood samples will be collected for these studies. The cells from the sample and the products derived from them if any may be stored and used in the laboratory till it is exhausted.

I have been informed that the possible discomforts and risks attendant to this procedure includes some degree of pain from the insertion of the needle and that this is usually minimal. If any tests are performed on my sample, I will be told which tests have been performed and the results could be made available to me.

I will receive a copy of this consent. The records connected with their participation will be kept strictly confidential and my name will not be revealed in any publication that may arise from this study.

I can ask questions concerning the studies carried out on the samples collected from me. If any further questions concerning the research conducted persist, I may contact the concerned faculty in the Department of Haematology, Christian Medical College, Vellore.

I understand that I am free to withdraw consent at any time without any penalty or loss of benefits to which I may otherwise be entitled to. I understand that I will not be paid for participation in this study, but there will be no additional cost to me for evaluations necessary for this study. I have been informed that there will be no immediate benefit to me from the studies being performed.

In addition any data obtained from these samples may be used for any future studies planned by the Department of Haematology, Christian Medical College, Vellore.

If I require any further information I may contact Dr. Vikram Mathews Department of Haematology, Christian Medical College, Vellore, 632 004 at 0416-228 2352 (or) the Secretary of the Ethics committee office at 0416-228 4294.

I / (my child), hereby voluntarily give consent for the sample to be used for research purpose and I am willing for a sample to be collected and used as indicated above.

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Signature of Subject/Parent/Guardian Name Date

In case of minors, the nature of this study has been explained as fully as is possible given their age and verbal assent has been obtained.

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INVESTIGATOR'S OR SUB-INVESTIGATORS STATEMENT

I have offered an opportunity for further explanation of this procedure to the individual whose signature appears above.

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Note: The subject shall be given a copy of this consent form. A signed copy must be filled in the patient's file at the Department of Haematology, Christian Medical College, Vellore