Genomic and functional studies in leukemia patients exhibiting TCR γδ gene rearrangement

By

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[LIFE09201004016]

Tata Memorial Centre

Mumbai

A thesis submitted to the

Board of Studies in Life Sciences

In partial fulfillment of requirements

For the Degree of

DOCTOR OF PHILOSOPHY

of

HOMI BHABHA NATIONAL INSTITUTE

March, 2017

Homi Bhabha National Institute

Recommendations of the Viva Voce Committee

As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by Ms. Gauri Ravindra Mirji entitled "**Genomic and functional studies in leukemia patients exhibiting TCR γδ gene rearrangement**" and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

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Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to HBNI.

I hereby certify that I have read this thesis prepared under my direction and recommend that it may be accepted as fulfilling the thesis requirement.

Date: 08.03.2017
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Dr. Shubhada V. Chiplunkar
Guide

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Gauri Ravindra Mirji

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

Gauri Ravindra Mirji

List of Publications arising from the thesis

Journal

1. "Risk stratification of T-cell Acute Lymphoblastic Leukemia patients based on gene expression, mutations and copy number variation", Mirji G, Bhat J, Kode J, Banavali S, Sengar M, Khadke P, Sait O, Chiplunkar S., *Leukemia Research 45 (2016) 33–39.*

2. "TCR γδ+T-ALL patients exhibit differences in immune responses as compared to TCRαβ+T-ALL", Mirji G, Banavali S, Sengar M, Chiplunkar S. (manuscript under preparation)

3. "Role of γδ T cells in ALL patients undergoing allogenic bone marrow transplantation", Mirji G, Khattry N, Chiplunkar S. (manuscript under preparation)

Conference proceedings

1. "Anti-leukemic activity of γδ T cells in T-acute lymphoblastic leukemia patients", G. Mirji, S. Banavali, M. Sengar, S. Chiplunkar, European Journal of Cancer 54 (2016) S1–S72

2. "Anti-leukemic activity of γδ T cells in T-acute lymphoblastic leukemia patients", Gauri Mirji, Shripad Banavali, Manju Sengar, Shubhda Chiplunkar, Indian J Hematol Blood Transfus (Oct-Dec 2013) 29(4):191-277

3. Chicago 2014-30 years of γδ T cells, Meeting report of 6th International Gamma Delta T cell conference, Cell Immunol. 2015 Jul;296(1):3-9

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Presentations at conferences

Oral presentations

1. Anti-leukemic activity of γδ T cells in T-Acute Lymphoblastic Leukemia patients, oral presentation; Haematocon 2013, Hotel Taj Mahal Palace, Mumbai, 7th -10th November, 2013.

2. Anti-leukemic activity of γδ T cells in T-Acute Lymphoblastic Leukemia patients, oral presentation; 6th International Gamma Delta T cell conference, University of Illinois, Chicago, US, 16th -18th May 2014.

Poster presentations

1. Clonal T Cell Receptor γδ gene rearrangement in T-cell Acute Lymphoblastic leukemia patients: A potential prognostic biomarker, 1st Global Cancer Genomic Consortium held at ACTREC, Navi Mumbai, and 10th -12th Nov 2011.
2. Clonal T Cell Receptor γδ gene rearrangement in T-cell Acute Lymphoblastic leukemia patients: A potential prognostic biomarker, 7th Graduate Students Meet held at ACTREC, Navi Mumbai, 9th-10th Dec 2011.
3. Genomic and functional studies in T-Acute Lymphoblastic Leukemia patients exhibiting clonal T Cell Receptor γ and δ gene rearrangement, National Education Day 2012 symposium, at Mithibai College, Vile Parle (W), 1st November 2012.
4. Anti-leukemic activity of γδ T cells in T-Acute Lymphoblastic Leukemia patients, Tata Memorial Centre Platinum Jubilee Conference, at Tata Theatre, NCPA, Mumbai, 26th-28th Feb 2016.

Award received

Travel award was received from University of Illinois, Chicago to attend the 6th International Gamma Delta T cell conference- 2014 at University of Illinois, Chicago, US.

Gauri Ravindra Mirji

Dedicated to my parents..

ACKNOWLEDGEMENTS

“If you only say one prayer in a day, make it thank you.” -Rumi

"When I started counting my blessings, my whole life turned around.” - Willie Nelson

PhD was a life enriching experience to me. I not only learnt how to conduct research experiments but most importantly how to tackle the difficult situations in life with a positive attitude. There were many ups and downs but that’s how I learnt and gained many qualities. I wish to acknowledge all the people who have helped and taught me during my tenure.

First of all, I would like to thank my mentor and supervisor Prof. Shubhada V. Chiplunkar for giving me a chance to work in her lab and supporting me throughout my PhD. She has been a great inspiration to me to pursue my research career. She has always given valuable suggestions, encouragement and freedom to carry out experiments and explore novel ideas. Her moral support in difficult times helped me to regain the strength. She has been a real mentor who understood all personal and scientific difficulties and cared for smallest things.

I would like to thank my Doctoral Committee members for their valuable suggestions. I am thankful to Prof. M. Seshadri (former chairperson), Dr. Neelam Shirsat (present chairperson), Dr. Naveen Khattry and Dr. Dibyendu Bhattacharyya. I would like to specially mention Dr. Khattry for being very kind and helpful whenever I was in trouble.

The work would not have been possible without participation of leukemia patients and healthy volunteers. I would like to thank Dr. Shripad Banavali, Dr. Manju Sengar and Dr. Khattry for kindly providing me the blood samples of patients and for all the scientific discussions. I would also like to thank Ms. Krutika, Dr. Girish and Dr. Amey for collecting and sending the samples from TMH and Mr. Libin for sending the samples from ACTREC, which helped me to complete the accrual target. I am thankful to sample collection unit for collecting the blood of healthy volunteers. I am also thankful to Mrs. Sadhana Kannan for the help in statistical analysis of data.

I would like to thank Chiplunkar Lab members for constantly supporting me. I learnt many things from my seniors and they acquainted me with scientific techniques. I am thankful to Bhairav, Nirmala, Dimpu, Jaydeep, Atul, Pradeep, Babita, Swati, and Aparna. I am thankful

to my colleagues Sagar, Priyanka, Anjali, Asif, Rushikesh, Siddhesh, Sachin, Shalini and Sajad. I am thankful to Sajad for being a great friend and always helping me and listening to my tantrums. I would like to thank Trupti mam, Shamal mam, Rekha mam, Meena mam, Dakave sir, Desai sir, Sawant bhai and Ram for making lab atmosphere lively and for all scientific and technical help and troubleshooting and chit chatting. I am thankful to Dr. Kode for helping me in writing my first manuscript and carrying out experiments.

I am also thankful to all the support staff- Mrs. Preeta, Mrs. Maya, Mr. Dandekar, administration, accounts, security, and engineering departments for help in academic and non-academic work. I would like to thank imaging, flow cytometry, genomics, common instrument, and library facilities for providing the infrastructure.

Thanks to my batchmates (batch 2010) for the fun time that we had together in hostel, for the parties and picnics. I got to live my student life again because of all of you. I got a privilege to work for student's council of ACTREC and I learnt problem solving skills. Student community of ACTREC has been very supportive and helpful.

Lastly and most importantly I would like to thank my parents for giving me unconditional support and freedom to choose and pursue my career and believing in me. My family and relatives gave me the all the mental and emotional support. I am grateful to almighty Lord for everything that I experienced and for my existence.

"Let us rise up and be thankful, for if we didn't learn a lot today, at least we learnt a little, and if we didn't learn a little, at least we didn't get sick, and if we got sick, at least we didn't die; so, let us all be thankful." ~ Buddha

Gauri Ravindra Mirji

CONTENTS

Synopsis

Homi Bhabha National Institute

SYNOPSIS OF Ph. D. THESIS

1. **Name of the Student:** Gauri Ravindra Mirji
2. **Name of the Constituent Institution:** Tata Memorial Centre, ACTREC
3. **Enrolment No. :** LIFE09201004016
4. **Title of the Thesis: "**Genomic and functional studies in Leukemia patients exhibiting TCR γδ gene rearrangement."
5. **Board of Studies:** Life Science

SYNOPSIS

Title: "Genomic and functional studies in Leukemia patients exhibiting TCR γδ gene rearrangement"

Introduction

T cell acute lymphoblastic leukemias (T-ALLs) are aggressive hematologic tumors resulting from the malignant transformation of T cell progenitors arrested at various stages of development. ALL accounts for 10%–15% of paediatric and 25% of adult ALL cases and is characteristically more frequent in males than females [1]. T-ALL is a phenotypically and clinically heterogeneous disease. Major improvements have been made in ALL treatment, with successful long-term survival rates of approximately 80% over the past four decades. Despite the significant success rate, the remaining 20% of patients still present treatment failure [2]. The risk-based treatment assignment should be utilized to treat patients, who have

Version approved during the meeting of Standing Committee of Deans held during 29-30 Nov 2013

a high frequency of treatment failure. Risk-based treatment assignment requires the availability of prognostic factors that reliably predict outcome [3].

Earlier studies reported from our laboratory on T-cell Acute Lymphoblastic Leukemia (T-ALL) patients, using a sensitive PCR-coupled Heteroduplex assay, identified clonal TCR γ and δ junctional gene rearrangement status of T-ALL patients as a prognostic marker [4]. Clinically, survival probability was higher in TCRγδ+ T-ALL patients over TCRαβ+ T-ALL patients when TCR γ and δ gene rearrangement was considered as prognostic variable [5, 6].

γδ T cells account for 1–10% of T lymphocytes in the peripheral blood of healthy adults. In contrast to αβ T cells, peripheral blood γδ (Vγ9Vδ2) T cells do not see processed antigenic peptides presented by major histocompatibility complex molecules, but rather recognize heat shock proteins and non-peptide phosphoantigens such as isopentenyl pyrophosphate (IPP) and its structural analogs amino bisphosphonates [7, 8]. Human γδ T cells kill a vast repertoire of tumor cell lines and fresh tumors such as breast, oral, renal, melanoma, leukemia and lymphoma [9, 10]. Their anti-leukemic role was initially suggested by the observation that showed increased reconstitution of donor Vδ1 T lymphocytes, post transplantation that correlated with a better prognosis [11]. The Vδ1 T cell population was also predominant in newly diagnosed leukemia patients, suggesting that a γδ T cell-based immune response indeed occurs against primary leukemia.

Hypothesis

Earlier study from our lab has demonstrated that TCR γδ+T-ALL patients have significantly higher survival probability over TCR αβ+T-ALL patients. It was therefore hypothesized that TCR γδ clonal leukemic blasts may be genotypically, phenotypically and functionally

different from TCR αβ clonal blasts. The immune scenario may be different in patients harboring these clonal blasts and would explain the survival advantage of TCR γδ+T-ALL patients over TCR αβ+T-ALL patients. It is further proposed that in ALL patients undergoing allogeneic bone marrow transplantation, γδ T cells may reduce chances of Graft versus Host Disease (GvHD) and relapse.

Aims and Objectives

1. To study the immune scenario in T-ALL patients and analyze the functional role of γδ T cells against leukemic blasts
2. Genomic studies in T-ALL patients exhibiting TCRγδ and TCRαβ clonality
3. Comparison of immunophenotype and cytotoxicity of γδ T cells in TCR γδ and αβ clonal patients
4. Role of γδ T cells in ALL patients receiving allogeneic bone marrow transplantation

Material and Methods

Study group

Heparinized peripheral blood samples were collected from patients with newly diagnosed T-ALL (n=150) and patients in remission (n=32), attending the out-patient department at Tata Memorial Hospital (TMH), Mumbai, India. Peripheral blood samples were also collected from healthy individuals (n=50). Informed consent was obtained from all patients and healthy volunteers included in the study. The study was approved by Institutional Ethics Committee of Tata Memorial Centre, Mumbai. Leukemic blasts were separated using ficoll-hypaque density gradient.

Enrichment and purification of γδ T cells from PBLs of healthy individuals

Peripheral Blood Lymphocytes (PBLs) were isolated from healthy individuals (HI) by Ficoll Hypaque density gradient centrifugation and were enriched by HDMAPP (1-Hydroxy-2-methyl-2-buten-4-yl 4-diphosphate) (Echelon Bioscience, Salt Lake City, UT) and recombinant IL-2 (rIL-2) as described by Hintz et al. [12]. γδ T cells were purified by immunomagnetic purification, using TCR-γδ Microbead Kit (Miltenyi Biotech, Germany).

Immunophenotyping of T-ALL patients:

Immunophenotype of T-ALL patients at diagnosis and remission and in HI were studied using multiparametric flow cytometry. Multiple surface as well as intracellular markers were studied simultaneously such as, γδ T cell subsets (Vδ1, Vδ2), activation markers (CD25, CD69) and Memory markers (central memory CD45-CD27+, effector memory CD45-CD27-, naïve CD45+CD27+, TeMRA CD45+CD27-), CD4, CD8, CD56, NKG2D, CD16b, and Perforin.

Confocal Microscopy

To study the immune synapse between leukemic blasts and γδ T cells, time lapse and conjugate formation experiments were carried out. Leukemic blasts from patients were kept untreated or treated with 100 μM Zoledronate for 16-18 hours and then co-cultured with γδ T cells for 30 min. These cells were labelled with different dyes for e.g. LysoTracker red (50nM), or Cell Tracker Blue CMAC (5μM), or Calcein AM (1mM) or Phalloidin TRITC or conjugated antibodies against immune synapse molecules (CD166, P-Tyr, LFA-1, Dynamin-2). Conjugates were examined using a Zeiss 780 Meta confocal laser-scanning microscope (Carl Zeiss, Jena, Germany).

Cytotoxicity assay

Cytotoxic potential of γδ T lymphocytes was analysed against leukemic blast cells used as a target in ^{51}Cr release assay. T-ALL patient's blast cells were either treated with zoledranate or kept untreated. 1 x 10^6 leukemic blast cells from T-ALL patients were labeled with 100 μCi of ^{51}Cr (sodium chromate, Amersham, UK). γδ T lymphocytes were titrated at different effector: target ratios (40:1, 20:1, 10:1, 5:1 and 2.5:1) in triplicates and added to ^{51}Cr-labeled tumor targets (5 x 10^3). Leukemic blasts were co-cultured with γδ T lymphocytes for 4 hours at 37°C in 96-well plates (Nunc, Denmark). After 4 hours, radioactive chromium release was measured using 1470 Wallac automated gamma counter (Perkin-Elmer, Downers Grove) and expressed as counts per minute (cpm).

Clonality of γδ T cells from T-ALL patients:

DNA was extracted from leukemic blasts of T-ALL patients using Qiagen DNeasy kit. DNA was quantitated by NanoDrop (ND1000 software) and quality of extracted DNA was checked by 0.8% agarose gel electrophoresis. PCR for TCR γ and δ gene rearrangement was carried out. It was further analysed for clonality by heteroduplex assay. For heteroduplex analysis, 20 μl of amplified PCR product positive for γδ PCR was heated at 95°C for 5 minutes and rapidly cooled to 0°C and left at this temperature for 1 hr in a thermal cycler. The samples were run on 6% non-denaturing polyacrylamide gel (29:1, acrylamide: bisacrylamide) in Tris-borate-EDTA buffer at constant voltage of 110 volts. Heteroduplex patterns were visualized by silver staining.

cDNA Microarray

Total RNA was isolated from leukemic blasts of TCRαβ+ T-ALL and TCRγδ+ T-ALL patients using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) as per the standard protocol. Microarray experiments were performed through customized service using Agilent platform

(Agilent Technologies, Palo Alto, CA, USA). Data was analysed using GeneSpring GX (v9.0) software (Agilent Technologies, Bengaluru, India) and TM4 Microarray Multiexperiment Viewer (T_MeV). Differentially regulated genes were clustered using hierarchical clustering. Fold change was calculated as log base 2. P value was calculated using volcano Plot.

Quantitative Real Time PCR

Microarray data was validated using quantitative real time PCR (qRT PCR). Reactions were carried out on ABI Prism 7900HT sequence detection system (Applied Biosystems). Relative mRNA level expression was quantitated using β-Actin (*ACTB*; NM_001101) as housekeeping gene. Fold changes in target gene expression were normalized to housekeeping gene via the published comparative $2^{-\Delta\Delta Ct}$ method [13].

Droplet Digital PCR

Droplet Digital PCR (ddPCR) was carried out using the QX200 Droplet Digital PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Samples were analysed for mutations in *NOTCH1* (pL1600P), *IKZF3* (pL162R) and *BRAF* (pV600E) genes and CNV in *P53*, *CDKN2A* and *CDKN2B* genes. Droplet generation and droplet reading for ddPCR were carried out according to the manufacturer's instructions using Bio-Rad reagents. *RPP30* was used as reference gene in CNV analysis. Results were analysed using QuantaSoft Software (Bio-Rad) and represented as copy numbers and concentration of mutation (copies/µl).

Statistical Analysis

Graphs were plotted using Prism 5 software (GraphPad Software, Inc, CA, USA). Overall survival (OS) and disease free survival (DFS) were evaluated according to Kaplan–Meier

survival analysis using SPSS Graduate Pack 21.0 software (SPSS Inc, Chicago, IL, USA). P values were assessed using log-rank test. $P<0.05$ was considered as statistically significant.

Results

Clonality of leukemic blasts from T-ALL patients

Out of 70 T-ALL patients, 29 patients were TCR γδ clonal, while 41 patients were found to be TCR αβ clonal. Amongst TCR γ gene family, VγI-Jγ1.3/2.3 sequences were most utilized (60%) while from TCR δ repertoire Vδ1-Jδ1 sequences were preferentially rearranged (27.14 %).

Differential regulation of genes within two T-ALL subtypes

Gene expression profiles of TCR γδ+T-ALL versus TCR αβ+T-ALL were studied using Agilent's whole human genome 4x44k gene array. Unsupervised hierarchical tree distinguished two subsets of T-ALL patients i.e. TCRγδ+T-ALL and TCRαβ+T-ALL, showing differential regulation of genes based on 2 fold change in gene expression with $p<0.05$. These genes were found to be involved in the pathways related to cluster of differentiation, haematopoietic cell lineages, toll-like receptors, cytokine signalling molecules, inflammatory responses and mainly apoptotic pathway, as observed by functional annotation of genes using Gene Ontology, DAVID Bioinformatics Resources and KEGG database.

Copy number variation and mutation analysis of T-ALL patients

Genes involved in disease biology and having equal expression in both the T-ALL subgroups, were further analysed for mutations and copy number variations. TCRγδ+T-ALL patients exhibited differential level of mutations for NOTCH1 and IKZF3; however BRAF mutations were detected at equal levels in both the subgroups. Although TCRγδ+T-ALL patients with

these mutations demonstrated improved disease-free survival (DFS) as compared TCRαβ+T-ALL patients, it was not statistically significant. Patients with homozygous deletion of CDKN2A/CDKN2B showed poor DFS in each subgroup. TCRγδ+T-ALL patients with normal/heterozygous deletion of CDKN2A/CDKN2B possess significantly better DFS over TCRαβ+T-ALL patients (p=0.017 and 0.045, respectively).

Immunophenotyping of T-ALL patients at diagnosis and remission

It was observed that T–ALL patients at diagnosis showed significant low number of naïve and central memory cells as compared to healthy individuals. Patients at remission showed significant high number of TemRA cells compared to patients at diagnosis. T-ALL Patients at remission showed higher expression of NK, Vδ2, Vδ1 T cells, activation markers (CD25, CD69) and perforin expression on CD3, Vδ2 and Vδ1 T cells as compared to patients at diagnosis as well as healthy individuals.

Ability of γδ T cells to kill leukemic targets and molecules involved

We observed that T-ALL patient's leukemic blasts were killed efficiently by effector γδ T lymphocytes. Zoledronate treated leukemic blasts show significantly higher % cytotoxicity over non-treated blasts. We studied the role of γδ TCR and NKG2D receptor in the killing of leukemic blast using cytotoxicity inhibition assay. % Cytotoxicity inhibition was calculated using the formula [1-(% Cytotoxicity after addition of antibody / % Cytotoxicity without addition of antibody)] X100. 51.7 % inhibition of cytotoxicity was observed after blocking the γδ TCR and 19.7 % inhibition after blocking NKG2D receptor. The % cytotoxicity was further inhibited by 57.7% when both γδ TCR and NKG2D receptors were blocked. Hence, γδ TCR and NKG2D receptor play important role in recognition and killing of leukemic blasts.

Immune synapse formation between γδ T cells and leukemic blasts

Phosphoantigen (Zoledronate) treated leukemic blasts showed higher expression of CD166 at the immune synapse over untreated blasts. γδ T cells showed increased LFA-1 and Dynamin-2 expression at immune synapse with zoledronate treated blasts. We observed increased expression of perforin at the immune synapse between zoledronate treated blast and γδ T lymphocytes over the untreated control. Zoledronate treated TCR γδ clonal leukemic blasts showed significantly increased F-actin polarization at the immune synapse with γδ T cells as compared to TCR αβ clonal leukemic blasts.

Differences in the cytotoxic potential and phenotypic characters of TCRγδ clonal Vs TCRαβ clonal patients

We observed higher % cytotoxicity of γδ T cells against zoledronate treated TCR γδ clonal leukemic blast cells (25%) as compared to zoledronate treated TCR αβ clonal leukemic blasts (15%) at 1:10 target (leukemic blasts): effector (γδ T cells) ratio. We also observed higher expression of perforin, activation markers, and higher number of γδ T cell subsets in TCR γδ clonal T-ALL patients as compared to αβ clonal T-ALL patients

Immune scenario in ALL patients undergoing allogenic Hematopoietic stem cell transplantation

Immunophenotype of patients undergoing allogenic BMT was carried out at various time points viz., at baseline, post BMT day 0, day 30, day 90, day 180 and day 365. We simultaneously collected respective donor's blood sample. We observed that patients with earlier WBC engraftment and chronic GVHD (cGVHD) showed better overall and relapse free survival. High level of terminally differentiated Vδ1 T cells at baseline is associated with delayed CMV reactivation in recipients. If donors and recipients had high Vδ1 TemRA cells

at baseline, then the respective recipients have significantly more chance of achieving remission post BMT.

Correlation of recipient's immunophenotype with clinical outcome post BMT

In a retrospective analysis, patients who exhibited acute GVHD, had significantly high Vδ2/NKG2D (p=0.03) on Day 180, post BMT. Patients with low Vδ2/NKG2D on Day 30 (p=0.028); high CD3 CM (p=0.048), and high NKT (p=0.048) on Day 90; and low CD3/CD16b on Day 180 (0.048) showed presence of chronic GVHD, hence these markers can be used as a predictor of cGVHD. Patients with early WBC engraftment show high NK (p=0.021) and high CD19 (p=0.021) on day 30; low CD3/NKG2D (p=0.008), low Vδ2/NKG2D (p=0.008), and low Vδ1/NKG2D (p=0.008) on Day 90, post BMT. Patients with CMV reactivation post BMT, have low Vδ2 TemRA on Day 90 (p=0.048).

Correlation of donor's immunophenotype with clinical outcome post BMT

Donor's immune cell composition affects the clinical outcome of recipients. If donors had high CD3 EM (p=0.028) and low Vδ1/NKG2D (p=0.028), recipients develop acute GVHD post BMT. Donors with high CD3 (p=0.032), high Vδ1 Perforin (p=0.035), and high Vδ2 Perforin (p=0.035) have significant correlation with treatment related mortality (TRM). Recipients show relapse, if donors had low NKT (p=0.034), low NK (p=0.045), and low CD3 Perforin (p=0.023). Recipients show significantly less CMV reactivation (p=0.022) if donors had high γδ T cells. Hence γδ T cells play important role to prevent CMV reactivation in recipients.

Summary and Conclusion

TCRγδ+T-ALL and TCRαβ+T-ALL are two distinct subgroups of T-ALL with different clinical outcome, and show differential regulation of genes. TCRγδ+T-ALL subgroup with

wild type/heterozygous copies of CDKN2A/B show significantly (p=0.017 and p=0.045, respectively) improved DFS as compared to TCRαβ+T-ALL. TCR γδ clonal leukemic blasts exhibit significantly better immune synapse formation with γδ T cells over TCR αβ clonal leukemic blasts. In allogenic BMT, recipient's immunophenotpe (Vδ2/NKG2D, CD3, and NKT) can be used as a predictor of chronic GVHD. Donor's immune cell composition (particularly Vδ1, Vδ2, NKT, and NK) can be used as a predictor of disease outcome. Recipients show significantly less CMV reactivation, if donors had high γδ T cells.

In conclusion, the present study underscores importance of TCR γδ clonality as a prognostic marker in T-ALL patients and explains the genomic and functional reasons for the same. Further, study also shows role of γδ T cell subsets in dictating clinical outcome of recipients post BMT.

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Publications in Refereed Journal:

a. Published: Mirji G, Bhat J, Kode J, Banavali S, Sengar M, Khadke P, Sait O, Chiplunkar S., Risk stratification of T-cell Acute Lymphoblastic Leukemia patients based on gene expression, mutations and copy number variation, Leukemia Research 45 (2016) 33–39.
b. Accepted: Nil
c. Communicated: Nil
d. Other Publications: -

Conference proceedings

- Anti-leukemic activity of γδ T cells in T-acute lymphoblastic leukemia patients, G. Mirji, S. Banavali, M. Sengar, S. Chiplunkar, European Journal of Cancer 54 (2016) S1–S72
- Anti-leukemic activity of γδ T cells in T-acute lymphoblastic leukemia patients, Gauri Mirji, Shripad Banavali, Manju Sengar, Shubhda Chiplunkar, Indian J Hematol Blood Transfus (Oct-Dec 2013) 29(4):191-277
- Chicago 2014-30 years of γδ T cells, Meeting report of 6th International Gamma Delta T cell conference, Cell Immunol. 2015 Jul;296(1):3-9

Public repository submission

- cDNA Microarray data deposited at Gene Expression Omnibus, NCBI, with accession no. GSE37389.

Conferences/workshops attended:

Oral presentations

- Anti leukemic activity of γδ T cells in T-Acute Lymphoblastic Leukemia patients, oral presentation; Haematocon 2013, Hotel Taj Mahal Palace, Mumbai, 7th -10th November, 2013.
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Poster presentations

- Clonal T Cell Receptor γδ gene rearrangement in T-cell Acute Lymphoblastic leukemia patients: A potential prognostic biomarker, 1st Global Cancer Genomic Consortium held at ACTREC, Navi Mumbai, and 10th -12th Nov 2011.
- Clonal T Cell Receptor γδ gene rearrangement in T-cell Acute Lymphoblastic leukemia patients: A potential prognostic biomarker, 7th Graduate Students Meet held at ACTREC, Navi Mumbai, 9th-10th Dec 2011.

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- Anti-leukemic activity of γδ T cells in T-Acute Lymphoblastic Leukemia patients, Tata Memorial Centre Platinum Jubilee Conference, at Tata Theatre, NCPA, Mumbai, 26th-28th Feb 2016.

Workshop

- Cancer Informatics – Analysis and Informatics of Microarray Data, National Centre for Cell Science & the Bioinformatics Centre, University of Pune, 13th-14th September, 2012.

Award received

Travel award was received from University of Illinois, Chicago to attend the 6th International Gamma Delta T cell conference 2014 at University of Illinois, Chicago, US.

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Date: 19/7/2016

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ABBREVIATIONS

APC	Allophycocyanin
αβ	Alpha beta
ADCC	Antibody dependent cellular cytotoxicity
Ag	Antigen
APC	Antigen presenting cell
BMT	Bone marrow transplantation
BrHPP	Bromohydrin pyrophosphate
CFSE	Carboxyfluorescein succinimidyl ester
CM	Central memory
cDNA	Complementary deoxy ribo nucleic acid
CMAC	7-amino-4-chloromethylcoumarin
CNV	Copy number variation
CMV	Cytomegalovirus
^{51}Cr	^{51}Chromium
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulphoxide
DFS	Disease free survival
ddPCR	Droplet digital PCR
EM	Effector memory
EDTA	Ethylenediaminetetraacetic acid
FPPS	Farnesyl pyrophosphate synthase
FCS	Fetal bovine serum
FBS	Fetal calf serum
FH	Ficoll-Hypaque
FITC	Fluoresecein isothiocyanate
γδ	Gamma delta
GVL	Graft versus leukemia
GvHD	Graft versus-host disease

HMBPP	1-Hydroxy-2-methyl-2-buten-4-yl 4-diphosphate
HDMAPP	(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
HSCT	Haematopoietic stem cell transplantation
HI	Healthy individual
HSPs	Heat shock proteins
HLA	Human leukocyte antigen
IS	Immune synapse
ICAM-1	Intercellular Adhesion Molecule 1
IPP	Isopentenyl pyrophosphate
LFA-1	Lymphocyte function associated antigen
LTR	LysoTracker Red
MACS	Magnetic assisted cell sorting
MHC	Major histocompatibility complex
MIC	MHC class I chain-related molecules
µg	Micro gram
µl	Micro litre
µM	Micro molar
ml	Mili litre
mAbs	Monoclonal antibodies
NK	Natural killer
NKT	Natural killer T cell
NKG2D	Natural-killer group 2, member D
NBP	Nitrogen-containing bisphosphonates
OS	Overall survival
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
rIL2	Recombinant interleukin 2

qRT PCR	Quantitative real time PCR
RFS	Relapse free survival
RT	Room temperature
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulphate
SMAC	Supramolecular activation clusters
T-ALL	T- acute lymphoblastic leukemia
TCR	T cell receptor
Tc	T-cytotoxic
TemRA	Terminally differentiated effector memory
TRITC	Tetramethylrhodamine B isothiocyanate
Th	T-helper
ULBP	UL-16 binding protein
WBC	White blood cell
Zol	Zoledronate

CHAPTER 1

Introduction

Normal T-cell development is a strictly regulated process in which hematopoietic progenitor cells migrate from the bone marrow to the thymus and differentiate from early T-cell progenitors toward mature and functional T cells [1]. During this maturation process, cooperation between a variety of oncogenes and tumor suppressors can drive immature thymocytes into uncontrolled clonal expansion and cause an aggressive hematological cancer , T-cell acute lymphoblastic leukemia (T-ALL). ALL accounts for 10%–15% of pediatric and 25% of adult ALL cases and is characteristically more frequent in males than females. Clinically, T-ALL patients show diffuse infiltration of the bone marrow by immature T cell lymphoblasts, high white blood cell counts, mediastinal masses with pleural effusions, and frequent infiltration of the central nervous system at diagnosis [2].

T cell transformation is a multi-step process in which different genetic alterations cooperate to alter the normal mechanisms that control cell growth, proliferation, survival, and differentiation during thymocyte development [2]. T-ALL is a phenotypically and clinically heterogeneous disease. Despite improved insights in T-ALL disease biology and comprehensive characterization of its genetic landscape, clinical care remained largely similar over the past decades and still consists of high-dose multi-agent chemotherapy potentially followed by hematopoietic stem cell transplantation. T-ALL is an aggressive hematologic cancer for which limited therapeutic options are available for patients with primary resistant or relapsed disease, underscoring the need for better treatment stratification protocols and for identifying more effective anti-leukemic drugs [3]. Even with aggressive treatment regimens, which are often associated with considerable side effects, clinical outcome is still extremely poor in a significant subset of T-ALL patients as a result of therapy resistance or haematological relapses [1]. Major improvements have been made in ALL treatment, with successful long-term survival rates of approximately 80% over the past four decades. Despite the significant success rate, the remaining 20% of patients still present

treatment failure [4]. Therefore, more effective drugs and better treatment stratification are urgently needed for the treatment of T-ALL [5].

As there is paucity of information on risk factors in Indian patients with ALL, all the patients are treated uniformly on ‘‘high risk arm’’ of MCP 841 protocol designed by National Cancer Institute, USA. Though this protocol has brought major improvement in the survival (58.4% DFS) over earlier rates, significant proportion of patients still relapse or succumb to death due to infection, hemorrhage or febrile neutropenia [6]. Earlier studies have reported an association between clinical features such as high WBC count, blasts count, lymphadenopathy, splenomegaly at presentation and significantly shorter survival in T-ALL patients [7]. Other investigators have also analysed molecular markers viz. abnormalities of 9p deletion/inactivation of $p16^{INK4a}$ and $p15^{INK4b}$ [8, 9], tal-1 deletions [10-12], translocation of bcr-abl [13], translocations involving t(11;14) [14], ALL-1 gene rearrangement [15], and HOX11L2 gene [16] as prognostic variables in survival of T-ALL patients.

Risk-based treatment assignment is utilized for children with non T-ALL so that those children who have a very good outcome with modest therapy can be spared more intensive and toxic treatment, while a more aggressive (and thus more toxic) therapeutic approach can be provided only to those patients who have a lower probability of long-term survival [5]. However, children with T-ALL are usually treated on the high risk arm of most ALL protocols. Risk-based treatment assignment requires the availability of prognostic factors that reliably predict outcome [17].

Earlier studies from our lab have demonstrated that 30% of Indian T-ALL patients exhibit clonal rearrangements of both TCR γ and δ genes and TCRγδ+ T-ALL is a distinct subgroup amongst Indian T-ALL patients [18]. We identified clonal TCR γ and δ junctional gene rearrangement status of T-ALL patients at diagnosis as a prognostic marker and predictor of response to chemotherapy [19]. Disease free and event free survival was better in

TCRγδ+ T-ALL patients over TCRαβ+ T-ALL patients when γ and δ gene rearrangement was considered as prognostic variable [19, 20]. This observation provided an impetus to investigate how TCRγδ+ T-ALL differs biologically from TCRαβ+ T-ALL subgroup.

The T-cell antigen receptor (TCR) is a multichain receptor complex composed of a polymorphic heterodimer of two polypeptide chains αβ or γδ, which are both non-covalently associated with the signal-transducing monomorphic CD3 protein complex. TCR αβ or γδ molecules are expressed by mutually exclusive populations of T lymphocytes which differ in their immunophenotype, ontogeny, tissue tropism, TCR gene usage and functions [21]. γδ T cells are immunosurveillance cells, comprising 1-10 % of circulating lymphocytes in humans. In contrast to αβ T cells, peripheral blood γδ (Vγ9Vδ2) T cells do not see processed antigenic peptides presented by major histocompatibility complex molecules, but rather recognize heat shock proteins and non-peptide phosphoantigens such as isopentenyl pyrophosphate (IPP) and its structural analogs amino bisphosphonates [22, 23]. Human γδ T cells kill a vast repertoire of tumor cell lines and fresh tumors such as breast, oral, renal, melanoma, leukemia and lymphoma [24]. γδ T cells are involved in the immune defense against various leukemic cells and notably chronic myeloid leukemia cells and AML [25-30]. Their anti-leukemic role was initially suggested by the observation showing that an increased reconstitution of donor Vδ1 T lymphocytes, post transplantation, correlated with a better prognosis [25, 26, 31]. The Vδ1 T cell population is also predominant in newly diagnosed leukemia patients, suggesting that a γδ T cell-based immune response indeed occurs against primary leukemia [27].

T cell receptor engagement by an APC induces the formation of a highly organized network of receptors and intracellular signaling molecules [32], accompanied by a profound reorganization of the actin cytoskeleton [33] and extensive remodeling of surface proteins and lipids [34, 35]. The formation of this network, known as the immunological synapse, is a multistep process involving lateral movement of proteins along the plane of plasma

membrane that eventually coalesces at the contact area between the T cell and the APC [36-38]. The synapse is organized into two differentiated concentric rings or supramolecular activation clusters (SMAC) [36], termed the central SMAC, which contains the TCR, CD28, CD4, and associated signaling molecules [32, 39], and the peripheral SMAC (p-SMAC), which contains the adhesion LFA-1 and ICAM molecules and the cytoskeletal molecule talin [32, 40]. Ag-stimulated human Vγ9δ2 T cells conjugate to, and perform molecular transfer from, various tumor cell targets. The molecular transfer appears to be linked to IS establishment, evolves in a dose-dependent manner in the presence of either soluble or cellular Ag, and requires γδ TCR ligation, Src family kinase signaling, and participation of the actin cytoskeleton [41, 42].

Allogeneic haematopoietic stem cell transplantation (HSCT) is a potential curative treatment for malignant haematologic diseases [43]. Even though HSCT is a widely used and successful treatment for patients with life-threatening conditions, it is still a procedure associated with high transplant-related mortality and morbidity due to the toxicity of the preparative regimen together with the post-transplant immunodeficiency period predisposing the patient to potentially fatal viral, bacterial and fungal infections. The major advantages of immunotherapy with innate lymphocytes compared with MHC-restricted αβ T cells are that they can kill tumor cells without prior exposure and do not induce graft versus-host disease (GVHD) [44]. Human γδ T cells not only recognise microbial antigens, but are also capable of exerting significant MHC unrestricted activity against a broad spectrum of tumor cells in vitro, especially haematological neoplasia [45-47]. Clinical data is available suggesting an enhanced GVL effect and survival advantage from high levels of γδ T cells observed post-transplant in the patients. A significant lower incidence of infections with a total absence of bacterial infections in patients with elevated γδ T cells compared to patients with low or normal numbers was demonstrated [48-51].

CMV infection, one of the most common infectious complications in allograft recipients, is associated with a rapid, dramatic, and long-lasting expansion of peripheral γδ T cells. γδ T cells may represent a first-line defense mechanism against CMV infection in a person whose αβ T cell response has been weakened by immunosuppression [52]. Reactivation of human cytomegalovirus (CMV) after allogeneic stem cell transplantation is associated with the in vivo expansion of $V\delta2^-$ γδ T cells that react against CMV-infected cells, and such expansion is correlated with clearance of the virus [53]. Vδ1 cells reside within epithelial tissues, especially at sites of cytomegalovirus (CMV) replication. It was reported that patients who experienced CMV reactivation showed significant expansion of $V\delta1^+$ subset and contained more terminally differentiated cells, suggesting that this subset was actually fighting the virus in vivo [51]. Hence γδ T cells can provide critical antiviral and antileukemia effects after HLA-haploidentical hematopoietic transplants depleted of αβ T cells.

Hypothesis

Earlier study from our lab has demonstrated that TCR γδ+T-ALL patients have significantly higher survival probability over TCR αβ+T-ALL patients. It was therefore hypothesized that TCR γδ clonal leukemic blasts may be genotypically, phenotypically and functionally different from TCR αβ clonal blasts. The immune scenario may be different in patients harboring these clonal blasts and would explain the survival advantage of TCR γδ+T-ALL patients over TCR αβ+T-ALL patients. It is further proposed that in ALL patients undergoing allogeneic bone marrow transplantation, γδ T cells may reduce chances of Graft versus Host Disease (GvHD) and relapse.

Aim

In the present study we aimed at investigating the anti-leukemic role of γδ T cells in T-ALL patients at diagnosis and after BMT. The study further aimed at understanding the immune scenario generated by TCRαβ clonal and TCRγδ clonal leukemic blasts.

Objectives

1. To study the immune scenario in T-ALL patients and analyze the functional role of γδ T cells against leukemic blasts
2. Genomic studies in T-ALL patients exhibiting TCRγδ and TCRαβ clonality
3. Comparison of immunophenotype and cytotoxicity of γδ T cells in TCR γδ and αβ clonal patients
4. Role of γδ T cells in ALL patients receiving allogeneic bone marrow transplantation

CHAPTER 2

Review of Literature

2.1 Leukemia

Leukemia is a heterogeneous hematologic malignancy originating from a multipotent hematopoietic stem cell. It ranks among the commonest cancers in childhood and is characterized by excessive proliferation and differentiation block. Abnormal cells, which successfully undergo the transformation process, then efficiently settle in bone marrow and peripheral blood, gradually displacing normal cells on account of their exceptional phenotype manifested by e.g. excessive proliferation or resistance to apoptosis. This phenomenon is initiated in a single cell and is followed by acquisition of additional mutations and rearrangements during cell division [54]. Leukemia is classified in two main types i.e. acute or chronic based on course of disease progression and these types can be further subdivided according to cell lineage involvement i.e. lymphoid or mylogenous (Figure 2.1).

Acute lymphoblastic leukemia is thought to originate from various important genetic lesions in blood-progenitor cells that are committed to differentiate in the T-cell or B-cell pathway, including mutations that impart the capacity for unlimited self-renewal and those that lead to precise stage-specific developmental arrest [55, 56]. In some cases, the first mutation along the multistep pathway to overt acute lymphoblastic Leukemia might arise in a haematopoietic stem cell possessing multilineage developmental capacity [57]. The cells implicated in acute lymphoblastic leukemia have clonal rearrangements in their immunoglobulin or T-cell receptor genes and express antigen-receptor molecules and other differentiation-linked cell-surface glycoproteins that largely recapitulate those of immature lymphoid progenitor cells within the early developmental stages of normal T and B lymphocytes [3, 55, 56].

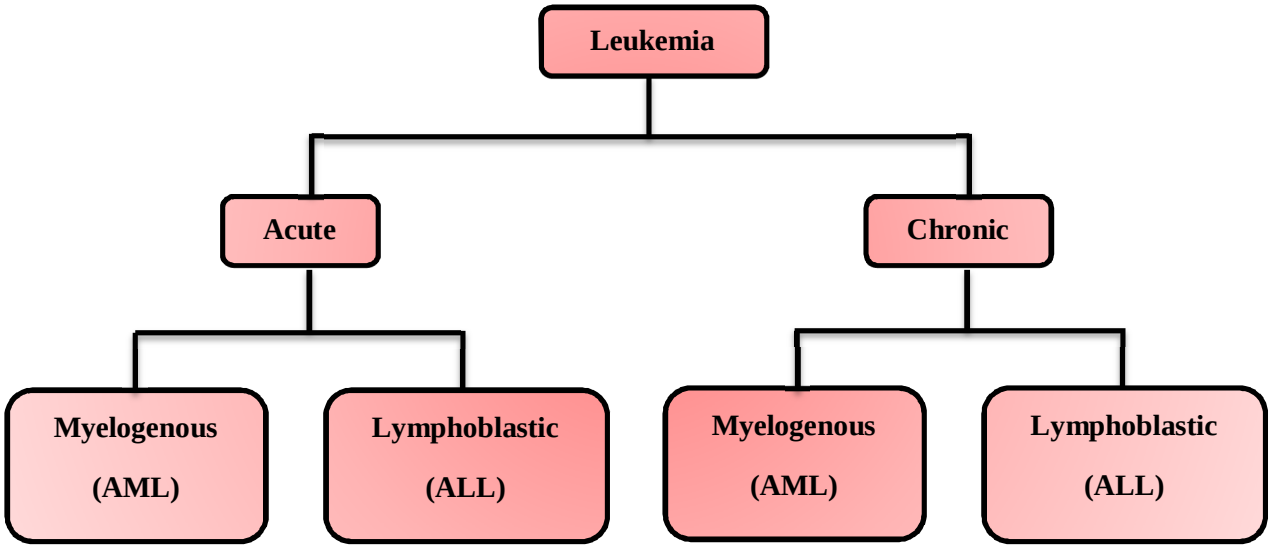


Figure 2.1 Classification of leukemia

T cell acute lymphoblastic leukemias (T-ALLs) are aggressive hematologic tumors resulting from the malignant transformation of T cell progenitors. T-ALL accounts for 10%–15% of paediatric and 25% of adult ALL cases [58] and is characteristically more frequent in males than females. Clinically, T-ALL patients show diffuse infiltration of the bone marrow by immature T cell lymphoblasts, high white blood cell counts, mediastinal masses with pleural effusions, and frequent infiltration of the central nervous system at diagnosis [1]. The prognosis of T-ALL in children and adolescents has improved in recent years due to intensified therapies, with 5 year relapse-free survival rates now in the range of 60%–75% [59]. Recurrent chromosomal translocations and intrachromosomal rearrangements are seen in T-ALL. These abnormalities typically juxtapose strong promoter and enhancer elements responsible for high levels of expression of T cell receptor genes next to a small number of developmentally important transcription factor genes, including HOX11/TLX1, TAL1/SCL, TAL2, LYL1, BHLHB1, LMO1, and LMO2 resulting in their aberrant expression in developing thymocytes [58].

2.2 Prognosis

Children aged 1–9 years have a better outcome than either infants or adolescents. Leucocyte count is a continuous prognostic variable, with increasing counts conferring a

poorer outcome, especially in patients with B-cell precursor disease. In T-cell acute lymphoblastic leukemia, a leucocyte count greater than $100x10^9$/L is associated with an increased risk of relapse in the CNS. The adverse prognosis previously ascribed to male sex has been abolished with enhanced treatment regimens. The Philadelphia chromosome, t(4;11) with MLL-AF4 fusion, and hypodiploidy (<44 chromosomes per leukemic cell) all confer a poor outcome, whereas hyperdiploidy (>50 chromosomes), TEL-AML1 fusion, and trisomy 4, 10, and 17 are associated with favourable prognosis [3]. An absolute lymphocyte count at the end of induction is related to favourable presenting features and good initial treatment response [60].

Risk-based treatment assignment requires the availability of prognostic factors that reliably predict outcome [17]. Earlier studies have reported an association between clinical features such as high WBC count, blasts count, lymphadenopathy, splenomegaly at presentation and significantly shorter survival in T-ALL patients [6, 7]. Molecular markers are also analysed viz. abnormalities of 9p deletion/inactivation of $p16^{INK4a}$ and $p15^{INK4b}$ [8, 9], TAL-1 deletions [10-12], translocation of BCR-ABL [13], translocations involving t(11;14) [14], ALL-1 gene rearrangement [15], and HOX11L2 gene [16] as prognostic variables in survival of T-ALL patients. The level of minimal residual disease (MRD) is an independent predictor of outcome and can define risk subgroups among clinically or genetically defined ALL subtypes [60].

There is paucity of information on risk factors in Indian patients with ALL, therefore all the patients are treated uniformly on ‘‘high risk arm’’ of MCP 841 chemotherapy protocol. In our previous studies we investigated the suitability of TCR γ and δ junctional gene regions as a prognostic marker in T-ALL [19, 20]. There were few earlier reports on the prognostic significance of surface expression of TCR γδ receptors [61, 62] and individual

TCR γ and δ junctional gene rearrangements in T-ALL patients and their use in detection of minimal residual disease (MRD) [10, 63-65].

Precise assessment of the risk of relapse in individual patients is essential to ensuring that intensive treatment is limited primarily to high-risk cases, thus sparing low-risk cases from undue toxicities. It is well recognized that effective treatment can abolish the adverse impact of many clinical and biologic features once associated with a poor prognosis [5].

2.3 Treatment

Treatment for acute lymphoblastic leukemia typically consists of a remission-induction phase, an intensification (or consolidation) phase, and continuation therapy to eliminate residual disease. Treatment is also directed to the CNS early in the clinical course to prevent relapse attributable to leukemic cells sequestered in this site. The drugs currently in use for these phases were developed and tested between the 1950s and 1970s, but efforts to identify new anti-leukemic agents have begun to intensify [3].

Optimal use of existing anti-leukemic agents and improved supportive care in contemporary clinical trials have improved the 5-year survival rate of childhood acute lymphoblastic leukemia (ALL) above 85% in developed countries. Further advances in survival and quality of life will require a better understanding of ALL pathobiology, the mechanisms of drug resistance, and drug disposition in the host, together with the development of innovative therapeutics. To this end, the advent of high-resolution genome wide analyses of gene expression, DNA copy number alterations, and epigenetic changes, and more recently, next-generation whole genome and transcriptome sequencing have provided new insights into leukemogenesis, drug resistance, and host pharmacogenomics, identified novel subtypes of leukemia, and suggested potential targets for therapy. The remarkable advances in biomedical technology, next-generation genome sequencing of leukemic cells and normal host cells, and high-throughput screening systems for new drugs

should bring the promise of personalized treatment with targeted agents to fruition, resulting in more effective and less toxic treatments for patients with ALL [5].

Despite our comprehensive understanding of the genomic landscape of human T-ALL, leukemia patients are still treated by high-dose multi-agent chemotherapy, potentially followed by hematopoietic stem cell transplantation. Even with such aggressive treatment regimens, which are often associated with considerable acute and long-term side effects, about 15% of pediatric and 40% of adult T-ALL patients still relapse, owing to acquired therapy resistance, and present with very dismal survival perspectives. Given the long-term side effects associated with intensive chemotherapy, risk stratification in future paediatric T-ALL treatment protocols should be further optimized based on our enhanced understanding of T-ALL disease biology.

Table 2.1: Opportunities for targeted therapy in T-ALL [66]

Pathway	Relevance	Therapies
NOTCH1	Activating mutations in the *NOTCH1* gene are present in over 50% of T-ALL cases .	• NOTCH1 inhibitory antibodies and stapled peptides (e.g., SAHM1) • γ-secretase inhibitors (e.g., compound E) • IGF1R inhibitors (e.g., BMS-536924) • SERCA inhibitors (e.g., thapsigargin) • HES1-signature antagonists (e.g., perhexiline)
IL7R/JAK/STAT	Activation of pathway in T-ALL by: • Gain-of-function mutations in *IL7R*, *JAK1*, *JAK3*, and *STAT5B* or loss of *PTPN2* • Overexpression of ZEB2 • Activation of NOTCH1 pathway	• JAK1/JAK2 inhibitors (e.g., ruxolitinib) • JAK3 inhibitors (e.g., tofacitinib) • STAT5 inhibitors
Mitochondrial apoptosis	Overexpression of BCL-2 is typical for immature T-ALL. IL7R/JAK/STAT signaling also leads to BCL-2 upregulation	• BCL-2 inhibitors (e.g., ABT-199)
PI3K/Akt/mTOR	Activation of pathway in T-ALL by: • Deletions or mutations in *PTEN* • Gain-of-function mutations in *Akt*, *PIK3R1*, or *PIK3CA* • IL7 stimulation	• mTOR inhibitors (e.g., rapamycin) • PI3Kγ/δ inhibitors (e.g., CAL-130) • dual PI3K/mTOR inhibitors (e.g., PI-103) • Akt inhibitors (e.g., A443654)
H3K27 demethylation	JMJD3 is overexpressed and oncogenic in T-ALL	• JMJD3 inhibitors (e.g., GSKJ4)
General transcription	Oncogenic driver genes are often associated with super-enhancers and are very strongly transcribed	• BRD4 inhibitors (e.g., JQ1) • CDK7 inhibitors (e.g., THZ1)
Cap-dependent translation	T-ALL cells depend on cap-dependent translation for their survival	• eIF4A inhibitors (e.g., silvestrol) • eIF4E inhibitors (e.g., 4EGI-1)

On the other hand, further reduction of chemotherapy can also be achieved by translation of molecular genetic findings into novel targeted therapies for the treatment of

human T-ALL. In that context, a variety of preclinical studies have reported promising therapeutic effects for particular small-molecule inhibitors targeting specific oncogenic pathways (Table 2.1). Hopefully, some of these novel therapeutic strategies can be implemented in daily clinical practice to complement low-dose chemotherapy [66].

2.4 Genetic landscape of T-ALL

T cell transformation is a multi-step process in which different genetic alterations cooperate to alter the normal mechanisms that control cell growth, proliferation, survival, and differentiation during thymocyte development. In this context, constitutive activation of NOTCH1 signaling is the most prominent oncogenic pathway in T cell transformation. Constitutive activation of NOTCH1 signaling is uniformly identified in over 50% of T-ALL patients [2]. Biallelic deletion or epigenetic silencing of the cyclin-dependent kinase inhibitor 2A gene (CDKN2A) are reported, which encodes both the tumor suppressors p16INK4A and p14ARF and whose inactivation neutralises both the TP53 and retinoblastoma pathways in most cases of T-cell and many cases of B-cell precursor acute lymphoblastic leukemia. Deletions of the CDKN2A locus in chromosome band 9p21, which encompasses the p16/INK4A and p14/ARF suppressor genes, are present in more than 70% of all T-ALL cases [2]. Thus, constitutive activation of NOTCH1 signaling cooperates with loss of p16/INK4A and p14/ARF in T-cell transformation and constitutes the core of the oncogenic program in the pathogenesis of T-ALL [1]. The NOTCH signaling pathway plays a critical role in cell lineage commitment decisions during development [2]. NOTCH1 mutations typically involve specific domains responsible for controlling the initiation and termination of NOTCH signalling [67]. NOTCH mutations are very frequent genetic alterations found in over 50% of T-ALL clinical cases, irrespective to their stage of the differentiation arrest [67, 68]. NOTCH1 signaling deregulation is considered to be crucial for the T cell leukemogenesis. Most common causes in NOTCH1 signaling deregulation are activating mutations clustered

in regions coding heterodimerization (HD) domain and proline, glutamic acid, serine, threonine-rich (PEST) domain [69], whereas HD mutations seem to enable the ligand-independent NOTCH cleavage resulting in the constitutive activation of the NOTCH protein [70], PEST domain mutations are thought to stabilize the structure and prolong the half-life of the active NOTCH1 [71]. Aberrant NOTCH1 activation in T-ALL is suggested to promote deregulated proliferation and prevent apoptosis [72].

Genes encoding transcriptional regulators of T-cell development and maturation are potential targets of T-ALL therapy. The IKAROS family, an important group of transcription factors in hematopoietic lineages, encodes a group of zinc-finger DNA-binding proteins essential for normal lymphocyte development [73-75]. The AIOLOS transcription factor, encoded by the IKZF3 gene, is necessary to control lymphocyte differentiation, proliferation and maturation [76]. AIOLOS is an IKAROS family member that was first described in committed lymphoid progenitors and was strongly upregulated as these progenitors become restricted into T- and B-lymphoid pathways [74]. Previous studies have shown that AIOLOS controls T and B lymphocyte apoptosis by regulating Bcl-xL [77] and also regulates cell death in T cells by controlling Bcl-2 expression and cellular localization [78]. Deregulated AIOLOS expression has been associated with leukemia and lymphoma in human patients [79-81]. Deletions were also detected in other B-cell developmental genes, such as TCF3 (E2A), EBF1 (EBF), LEF1, IKZF1 (Ikaros), and IKZF3 (Aiolos) [3].

In the past 5 years studies have included use of next-generation sequencing (NGS) techniques to identify all inherited and somatic genetic alterations [82-85]. NGS encompasses a range of techniques that enable the sequencing of hundreds of thousands of nucleic acid molecules simultaneously. In order of complexity, these approaches include sequencing of gene panels, exome sequencing, transcriptome (expressed RNA) sequencing and whole-genome sequencing (WGS), which are complementary approaches [86]. Exome-sequencing

and whole genome sequencing have found that genetic mutations on genes including NOTCH1, F-box and WD repeat domain containing 7 (FBXW7), Ras, PHD finger protein 6 (PHF6) and Janus kinase 1 (JAK1), MED12 are high-risk markers in patients with T-ALL [86-89]. These all studies highlight the underlying complexity of the genomic landscape of T-ALL, and the need of larger integrative studies in well-defined cohorts to increase understanding of the biological mechanisms that contribute to T-ALL and its various subtypes [89].

2.5 TCR gene rearrangements in T-ALL

Two distinct types of TCR can be discerned: the classical TCR αβ and the alternative TCR γδ, which are expressed on ≥85% and ≤15% of blood T-lymphocytes, respectively. TCR αβ molecules consist of disulfide-linked TCR α and TCR β glycoproteins, whereas in TCR γδ molecules, the TCR γ and TCR δ proteins can either be disulfide-linked or non-disulfidelinked depending on the use of Cγ2 or Cγ1 segment-derived TCR γ chains, respectively. Each TCR chain consists of a constant (C) and a variable (V) domain. The latter is the actual antigen-recognizing part of the receptor and is encoded by a combination of variable (V), diversity (D), and joining (J) gene segments, that are coupled via gene rearrangement processes during early T cell differentiation. The D gene segments are only present in TCRB and TCRD genes. The various distinct combinations of V, (D), and J gene segments determine the so-called combinatorial repertoire of the TCR chains and molecules. Since the TCRA and TCRB genes contain a larger number of functional V, (D), and J gene segments than the TCRG and TCRD genes, TCR αβ molecules have a more extensive potential combinatorial repertoire than TCR γδ molecules. The actual TCR γδ combinatorial diversity is even more limited due to the preferential use of available V and J gene segments. For instance, ~85% of TCR γδ+ T-lymphocytes in peripheral blood (PB) express Vγ9-Jγ1.2-Cγ1 chains in combination with Vδ2-Jδ1-Cδ chains, whereas in ~60% of the thymocytes

TCR γδ molecules contain Vδ1-Jδ1-Cδ chains, linked to any of the TCR γ chains [90-94]. In the thymus, TCR γδ molecules are expressed by only a minor fraction (<1%) of the thymocytes. However, TCR γδ expression is found at higher frequencies in malignant counterparts of cortical thymocytes, i.e. in approximately 10% of T cell acute lymphoblastic leukemias (T-ALL). Nevertheless, TCR γδ+ T-ALLs constitute a relatively small ALL subgroup, representing only ~2% of all ALL cases [95].

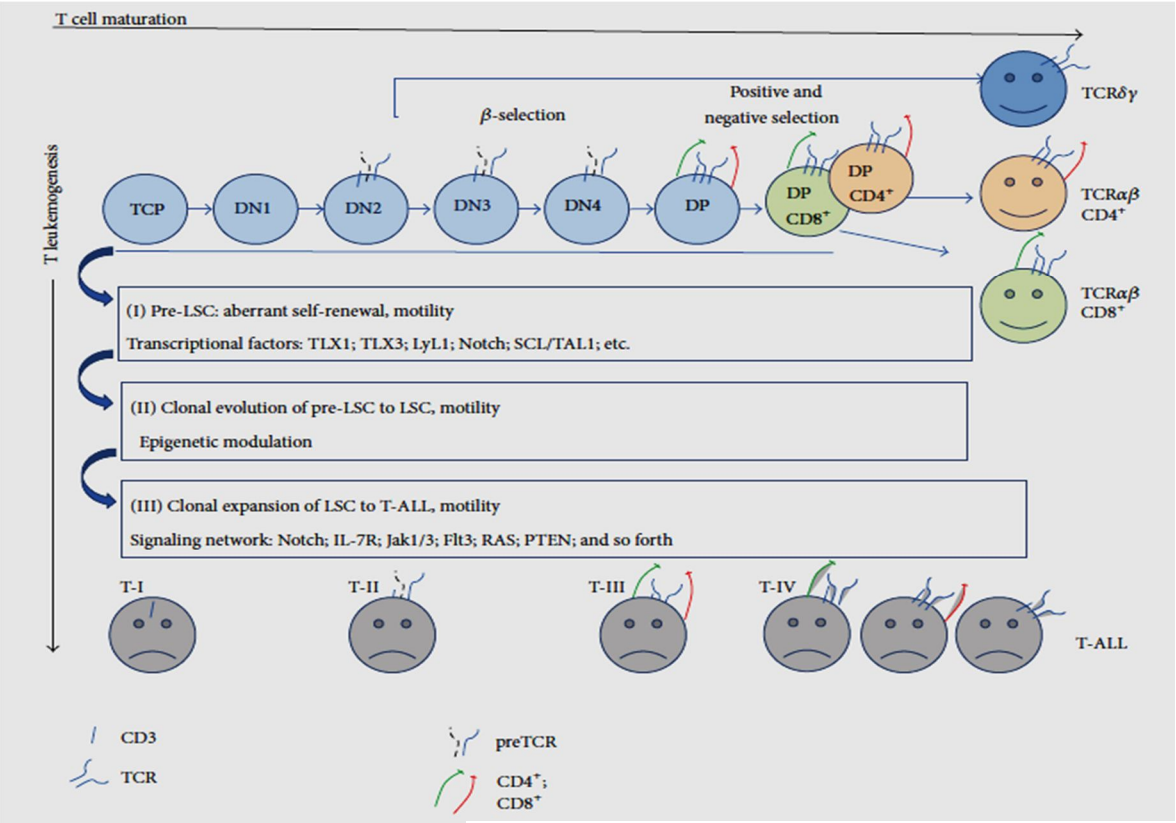
As shown in Table 2.2, the numbers of Vγ and Vδ gene segments are greatly limited in comparison to αβ T cells, and despite their relative low levels in peripheral blood, γδ T cells are found to be enriched at mucosal interfaces, such as the intestinal tract, that are heavily populated by largely non-pathogenic and symbiotic organisms [96].

Table 2.2: Comparative diversity of the α, β, δ and γ TCR chains [96, 97]

Vγ	~6 (5 from same family and 1 distantly related)
Vδ	~8 functional genes (only 3 commonly used-VD1, VD2 and VD3)
Vα	~70-80
Vβ	~52

T cells of TCR$\alpha\beta$ lineage constitute the bulk of T cell populations in lymphoid organs and recognize antigen-derived peptides bound to the molecules of a major histocompatibility complex, of classes I or II (MHC-I or MHC-II), on the surface of antigen-presenting cells. T cells of TCR$\gamma\delta$ lineage are generally not MHC-restricted and particularly play an important role in protection of the mucosal tissues from the external infection [72, 98, 99]. Maturation of self-tolerant T cells takes place in the thymus. The earliest T cells, lacking detectable CD4 and CD8 (CD4−CD8−) are referred to as double-negative (DN) cells. Later on, they start to

express both CD4 and CD8 (CD4+CD8+) and are denominated as double-positive (DP) cells. Finally, DP differentiates into single positive (SP) cells, either CD4+CD8− or CD4−CD8+, which leave to periphery. DN T cells are subdivided into four subsets (DN1-4), based on the presence or absence of other cell surface molecules, including CD117, the receptor for stem cell growth factor c-kit; CD44, an adhesion molecule; CD25, the α chain of the IL-2 receptor (IL-2R), determining the IL-2R affinity [5, 7]. In every DN stage, characteristic events of TCR rearrangements take place. DN1 thymocytes express only c-kit and CD44 (c-kit++ CD44+ CD25−), but once they encounter the thymic environment and become resident in the cortex, they express CD25 and proliferate, becoming DN2 thymocytes (c-kit++ CD44+ CD25+). During this stage, rearrangement and transcription of germ line Dβ and Jβ segments belonging to TCRγ and TCRδ gene locus begin. However, the TCRα locus does not rearrange, because the regions of DNA encoding TCRα genes are not yet accessible to the recombinase machinery. At the late DN2 stage, T cell precursors are fully committed to the T cell lineage and reduce expression of both c-kit and CD44. Cells in transition from the DN2 toDN3 (c-kit+ CD44− CD25+) stages continue rearrangement of the TCRγ, TCRδ, and TCRβ chains, start to express CD3, and make the first major decision in T cell development: whether to join the TCR$\gamma\delta$ or TCR$\alpha\beta$ lineage [72, 99-101].



Adapted from BioMed research international 2015;2015:750203

Figure 2.2: Hierarchical mutagenesis during T cell maturation causes different types of T-ALL [72]

As lymphoid leukemias and lymphomas can be considered as malignant counterparts of normal T cells in their various differentiation stages, most T cell malignancies contain rearranged T cell receptor (TCR) genes. Similar to other neoplasms, T cell malignancies are derived from a single malignantly transformed cell, implying that the TCR gene rearrangements within all the malignant cells are identical. Molecular analysis of TCR genes is therefore generally performed to prove or exclude clonality [102-105].

Several methodologies have been used to analyse TCR diversity, ranging from flow cytometric or PCR assessment of the relative usage of different Vβ families [106-108], to exhaustive sequencing [109]. Spectratyping, one of the broadly utilized techniques, measures TCR repertoire diversity based on variation in the lengths of RT-PCR products generated from the CDR3 region in each TCR Vβ family [110-112]. The CDR3 region encompasses the area of high diversity created by nucleotide insertions/deletions at the junction of the V, D

and J segments. Clonal T cell expansions or severe cytoreductions result in skewing in the proportional distributions of CDR3 of different lengths. One of the limitations of spectratyping is that rigorous analysis of the proportional distribution of CDR3 lengths relies upon the reproducibility of the spectratypes which requires large numbers of T cells [113]. Southern blot analysis was previously used technique which was considered as a reliable method, if proper probe/restriction enzyme combinations are used. Because Southern blot analysis of TCR genes is time-consuming and labor-intensive, PCR techniques are frequently being used as alternatives. The main disadvantage of the PCR-based detection of clonal TCR gene rearrangements, is the risk of false-positive results due to 'background' amplification of similar rearrangements in polyclonal reactive T lymphocytes. Therefore, PCR-based clonality assessment should include analyses that discern between PCR products derived from monoclonal and polyclonal cell populations. One such method is heteroduplex analysis, in which homo- and heteroduplexes resulting from denaturation (at 94°C) and renaturation (at lower temperatures) of PCR products are separated in non-denaturing polyacrylamide gels based on their conformation. After denaturation/renaturation, PCR products of clonally rearranged TCR genes give rise to homoduplexes, whereas in case of polyclonal cells heteroduplexes with heterogeneous junctions are formed. Heteroduplex PCR analysis of TCR gene rearrangements is a simple, rapid and cheap alternative to Southern blot analysis for detection of clonally rearranged TCR genes. Recently next-generation sequencing (NGS) based methods are being used for the comprehensive quantitative analysis of TCR repertoires at a clonal level [114].

2.6 γδ T cells

Our immune system responds to invading microbes such as viruses and bacteria and tries to eliminate the threat via two distinct but connected systems: the innate and the

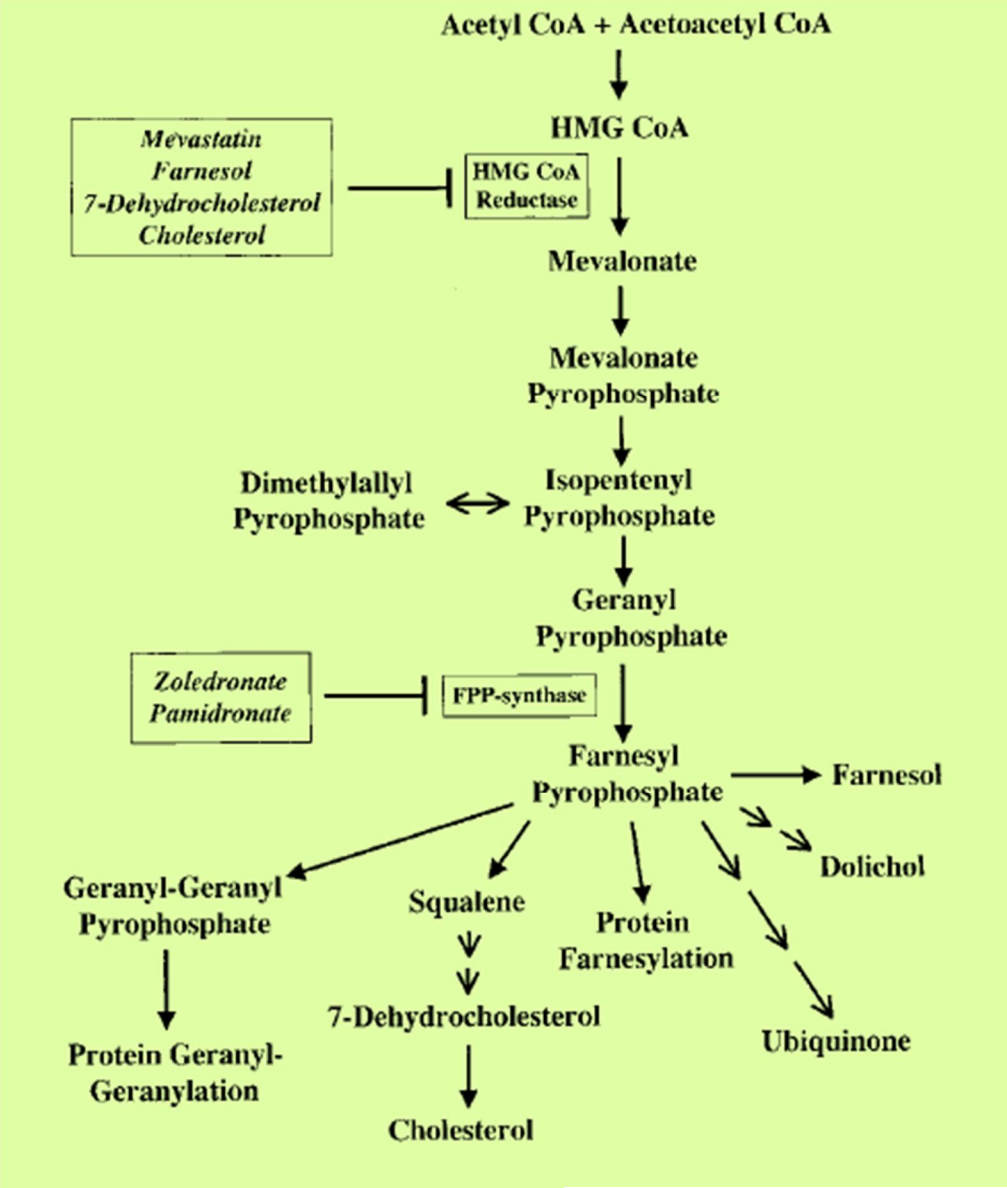
adaptive immune systems. Cells of the innate immune system patrol our organs and tissues in an effort to identify and eliminate threats with a quick but general response, which is similar for many different pathogens. This first line of defence also escalates the immune response by activating the adaptive immune system. The adaptive immune system consists of B cells, αβ T cells and γδ T cells. While αβ T cells perform all well-defined functions attributed to T cells, γδ T cells are also present together with αβ T cells in vertebrates. This suggests that each cell type performs unique functions and that both are necessary for host immune competence. Although γδ T cells and αβ T cells have similar effector functions, γδ T cells and αβ T cells are distinct in their antigen recognition and activation requirements, in their antigen-specific repertoire and effector function development [115].

There are several key differences between αβ and γδ T cells. First, although αβ T cells are primarily localized in secondary lymphoid organs, γδ T cells are relatively rare in lymphoid organs and, instead, predominate at epithelial surfaces [116, 117]. Second, αβ T cells recognize peptide ligands in the context of MHC class I and class II molecules, whereas γδ T cells do not recognize ligand in an MHC-restricted manner. Instead, γδ T cells recognize and respond to a broad range of antigens, including nonclassical MHC molecules, heat shock proteins, lipids and phosphoantigens [118]. Third, αβ T cells are naive upon exit from the thymus and acquire effector function in the periphery, but many γδ T cells acquire their effector fate during development in the thymus and exhibit limited plasticity after arrival in the periphery [119, 120]. Fourth, the role of various αβ T cell subsets in immune responses is relatively well understood, whereas much remains to be learned about the functional complexity of γδ T cells and its impact on immune responses [121].

γδ T cells account for 1–10% of T lymphocytes in the peripheral blood of healthy adults. Subpopulations of γδ T cells can be identified on the basis of T cell receptor (TCR) variable genes. The majority of blood γδ T cells express Vγ9 paired with Vδ2 [122, 123].

γδ TCR recognize molecules that are over-expressed in stress conditions. γδ T cells discriminate transformed tumor cells from healthy cells by the upregulation of self-antigens like heat shock proteins (HSP). The expressions of these proteins are increased in tumor cells due to higher metabolism and serves as endogenous danger signals. Studies from our laboratory have demonstrated that Vγ9Vδ2 T cells recognize HSP60 on oral tumor cells and have the ability to lyse autologous and allogenic oesophageal tumor targets via recognition of HSP60 and HSP70 [22, 124]. Vγ9Vδ2 T cells recognize phosphorylated small molecules, so-called phosphoantigens (pAg), which are intermediates of the microbial and eukaryotic isoprenoid biosynthesis pathway. Many bacteria produce the pAg (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), which stimulates human γδ T cells at pico- to nanomolar concentrations, whereas the eukaryotic pAg isopentenyl pyrophosphate (IPP) requires micromolar concentrations to activate γδ T cells. The selective sensing of microbial pAg by Vγ9Vδ2 T cells suggests that these cells play a pivotal role in anti-infective immune responses [125]. Vδ2+ γδ T cells are often regarded as sentinels against infection, whereas Vδ1+ γδ T cells are the most abundant subset in mucosal epithelia, forming part of the protective barrier against invading pathogens [126].

The concentration of IPP generated in normal cells is insufficient to activate γδ T cells, but higher concentrations accumulate upon cellular transformation which is associated with a dysregulated mevalonate pathway. In normal cells, the concentration of metabolites of the isoprenoid pathway, such as IPP, is too low to be sensed as a danger signal by Vγ9δ2 T cells. Deregulation of the isoprenoid pathway in some tumors leads to IPP over-production that is detected and considered as a tumor antigen by Vγ9δ2 TCR [127].



Adapted from JEM 2003;197:163-168

Figure 2.3: The mevalonate pathway for isoprenoid biosynthesis [128].

Vγ9δ2 T cells can be strongly and selectively activated in vitro by synthetic pAg such as bromohydrin pyrophosphate (BrHPP) or with nitrogen containing bisphosphonates, which induce the intracellular accumulation of IPP [128, 129]. Bisphosphonates, especially nitrogen-containing bisphosphonates (NBP) are widely used to treat postmenopausal osteoporosis and skeletal malignancies. NBP like Pamidronate, Alendronate, Zoledronate, etc. inhibit the key enzyme farnesyl pyrophosphate synthase (FPPS) of the mevalonate pathway, thereby upregulating the pool of endogenous IPP [130, 131]. Recently, a critical

role of the butyrophilin family member BTN3A1 (CD277) in the phosphoantigen-mediated activation of human Vδ2 cells has been discovered [132-134]. Even though the precise role of BTN3A1 is still under investigation, recent findings support intracellular sensing of prenyl pyrophosphates by BTN3A1 rather than its role on extracellular presentation of exogenous or endogenous pAg [135]. Once activated, Vγ9δ2 T cells mediate diverse effector functions. They produce cytokines such as TNF-α and IFN-γ, but can be also driven to secrete IL-4 or IL-17 [136, 137]. Vγ9δ2 T cells are unable to produce IL-2 in adequate amounts to sustain autocrine proliferation and therefore the addition of exogenous IL-2- or IL-2-producing CD4 T cells is required for proliferation [138].

Most γδ T cells express the activating NK receptor NKG2D (Natural Killer Group 2 Member D), which recognizes stress-inducible MHC class-I-related molecules including MHC class I chain-related antigen A/B (MICA/B). Concomitantly, γδ T cells can exert cytotoxicity against tumor cells through TCR- and/or NKG2D-dependent activation [139, 140]. In addition to cytokine production and cytotoxic activity, γδ T cells can exert regulatory and antigen-presenting functions; thus, they display a surprisingly large functional plasticity [127, 141-143].

Human class I-like molecules MICA and MICB were also suggested as natural antigens for human γδ T cells [144-147]. Interestingly, alterations in the expression of these ligands are induced by infection or tissue inflammation or stress, which can provide early danger-signal to initiate the activation of γδ T cells even in the absence of αβ+ T cells activation [148, 149]. The functions of γδ T cells in different pathophysiological conditions are driven by their tissue-specific distributions and tropism [150].

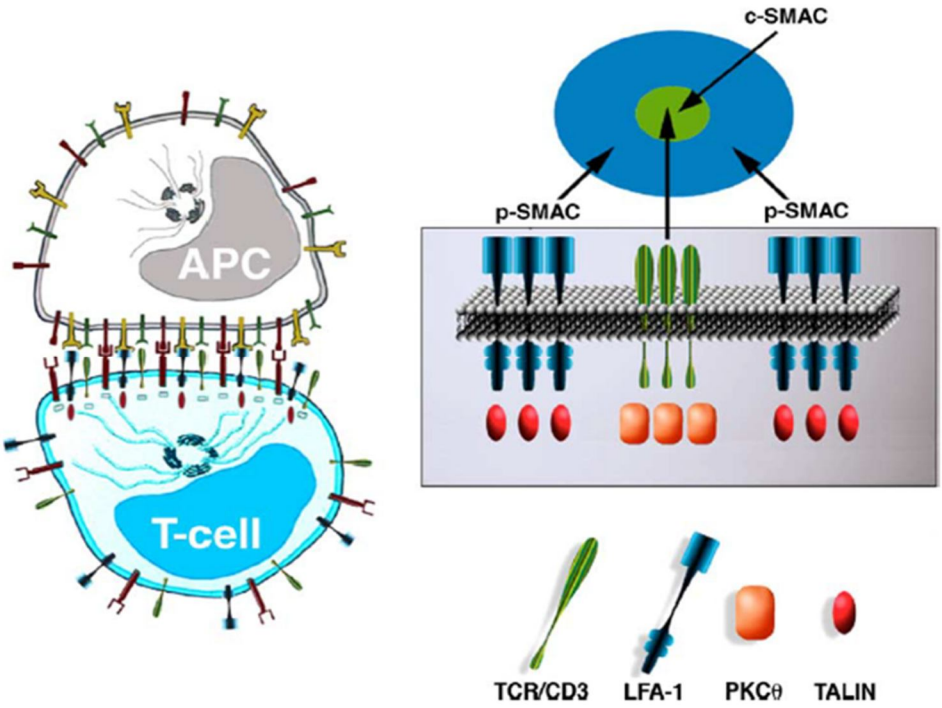
Since their serendipitous discovery some thirty years ago, γδ T cells have emerged as an evolutionarily conserved lymphocyte subset with great functional range, varying based on the species, tissue, and immunological milieu [151-154]. The differential contribution to

immunity by these cells is best illustrated by their variable abundance based on species and disease state. Effective functional differentiation of γδ T-cells is fine-tuned by the integration of signals from the γδ-TCR, and those arising from other environmental cues, such as cytokine and NOTCH receptor signals [155, 156].

2.7 Immune Synapse

Upon Ag recognition, B and T cells establish with target cells a tight and dynamic area of contact termed immunological synapse (IS). The specialized junction between a T lymphocyte and an antigen presenting cell, the immunological synapse, consists of a central cluster of T cell receptor surrounded by a ring of adhesion molecules. Immunological synapse formation is shown to be an active and dynamic mechanism that allows T cells to distinguish potential antigenic ligands [37]. IS formation is a multistep process with two major morphologically defined stages: the nascent or immature IS and the mature IS. In the nascent IS, the integrins are initially engaged in the centre of the contact area, and TCR are engaged in the periphery of the contact. This TCR ring of the nascent IS is the site of robust Lck and ZAP-70 activation. The mature IS has two major features. First, the pattern of the LFA-1 and TCR are inverted compared to the nascent IS, such that the TCR is clustered in the centre. Second, this central cluster of TCR–MHC interactions is stabilized [157].

Receptors at the interface between the T cell and its bound APC are highly organized both spatially and temporally. These highly organized molecular assemblies have been termed Supra-Molecular Activation Clusters (SMACs). The TCR and several protein kinases cluster in the central c-SMAC; LFA-1 and the cytoskeletal protein talin cluster in the peripheral p-SMAC (Fig. 2.4). It was demonstrated that the c-SMAC is the site of TCR engagement with its specific ligand on the APC [158].



Adapted from Seminars in immunology 2003;15:295-300

Figure 2.4: The spatial segregation of engaged receptors and intracellular proteins in distinct SMACs [158]

IS formation is more a consequence than a pre-requisite for TCR engagement and signalling [42]. The IS could augment and sustain the activation of signaling pathways [159, 160], favor polarized cytokine secretion [161], or rather enable TCR down-regulation [162]. TCR triggering led γδ T cells to establish an IS, to reorganize their actin cytoskeleton, and to initiate sustained intracellular signaling, enabling synaptic transfer of target cell molecules onto the γδ T cell [41]. γδ T cells spontaneously form a mature IS with THP-1 cells regardless of exogenous phosphoantigen addition, this latter is required to fully trigger γδ T cell cytokine responses [42].

2.8 Immunotherapy with γδ T cells

Vγ9Vδ2 T cells have rapidly emerged as an attractive therapeutic target for anti-tumor therapies. Indeed, they display a very efficient, non-MHC restricted lytic activity against a broad panel of tumors; they abundantly produce IFN-γ and can be easily expanded from

peripheral blood with agonist molecules. On the path to successful immunotherapy of hematopoietic tumors, γδ T cells offer great promise because of their human leukocyte antigen (HLA)–unrestricted targeting of a wide variety of leukemias/lymphomas [163]. Many clinical trials have been carried out based on the adoptive transfer of in vitro stimulated Vγ9Vδ2 T cells or on the in vivo stimulation of their activity using clinical-grade agonists [164, 165]. Malignant cells can have a dysfunctional mevalonate pathway, which can cause them to over-express IPP at levels capable of activating Vδ2 cells. Accumulation of mevalonate metabolites in tumor cells is a powerful danger signal that activates the immune response and may represent a novel target of tumor immunotherapy [128].

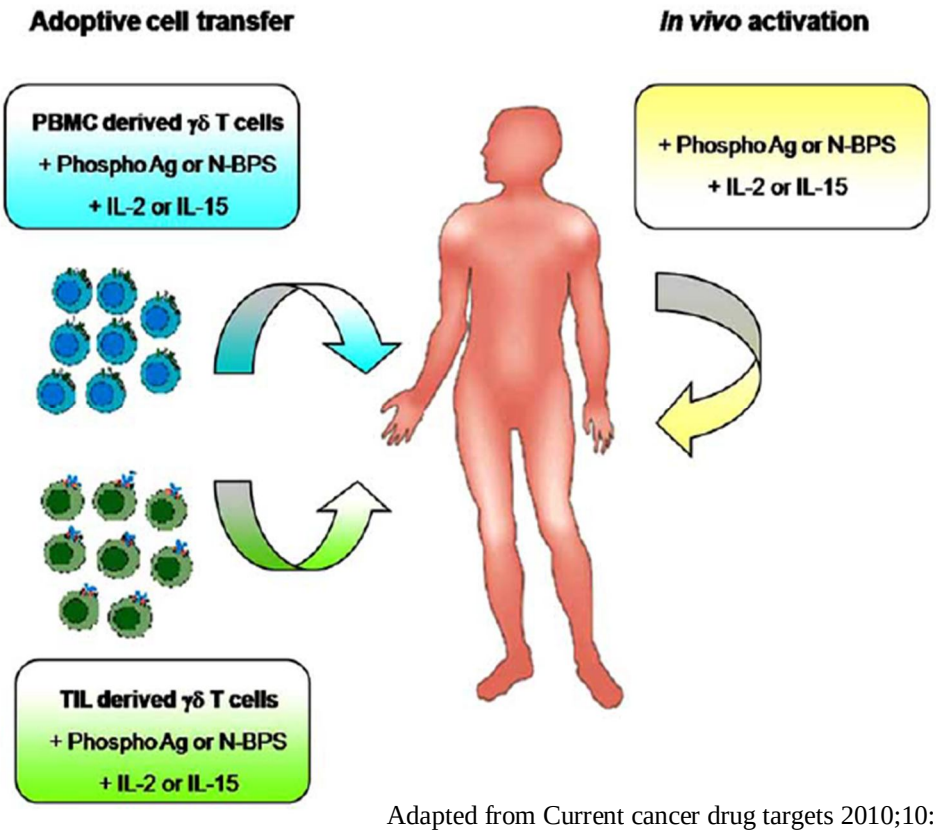
Human Vδ2 cells were found to kill a broad range of tumor cell lines derived from haematological and solid malignancies in both allogeneic and autologous settings. Vγ9Vδ2 T cells recognize bone marrow–derived tumor cells such as the non-Hodgkin B cell lymphoma line Daudi both in vitro and in a SCID animal model in vivo [166]. Vγ9Vδ2 T cells also recognize and kill the B cell lymphoma RPMI-8233 [167], the T cell lymphoma MOLT-4 [168] and the erythroleukemia line K562 [169]. Combination treatment utilizing Vγ9Vδ2 T cells along with chemotherapeutic agents and Zoledronate has been shown to induce an increase in the cytotoxic function of γδ T cells against solid tumor [23, 170]. The ability of γδ T cells to efficiently kill bisphosphonates treated colon cancer stem cells and ovarian cancer stem-like cells has also been reported [171, 172].

Following expansion on Zoledronate, Vγ9Vδ2 cells can be efficiently transduced to express CD19- and GD2-specific CARs with γ-retroviral vectors which displayed re-directed specificity toward CD19+ and GD2+ tumor targets, respectively. Chimeric antigen receptors (CARs) can be introduced into T cells and re-direct the T cell toward a specific antigen [173]. Genetic modification of T cells has clinical applications as adoptive transfer of CAR+ T cells with specificity for CD19 can lead to antitumor responses in patients with refractory B-cell

malignancies. The human application of CAR+γδ T cells is appealing given their inherent potential for antitumor effects and their apparent lack of alloreactivity [174, 175].

In patients, both positive and negative correlations have been made between clinical responses and tumor infiltrating Vδ2 cells. Adoptive cell transfer is an appealing approach for cancer immunotherapy. It has the potential to generate consistently large pools of tumor-reactive cells because the cells are stimulated outside the immunosuppressive environment of their tumor-bearing host. Furthermore, the cells can be harvested and used at the point at which they display optimal effector functions and migratory function [126]. Despite the ready expansion and cytolytic effector functions of γδ T cells demonstrated at the bench, attempts to harness this potential in the clinical setting have been disappointing due to the inevitable loss of these cells that is observed in many cancer patients [96].

Adoptive transfer of γδ T cells pre-stimulated with phosphoantigens or co-administration with aminobisphosphonates and IL-2 seems to be an attractive modality of current immunotherapeutic strategies with promising results [176]. Infusion of ex vivo activated and expanded γδ T cells in cancer patients have also shown anti-tumor activity [24, 177, 178]. Amino bisphosphonates or bromohydrin pyrophosphate (BrHPP) have been administered with low dose IL-2 in several clinical trials, to promote the activation and expansion of γδ T cells in vivo as immunotherapy for patients with lymphoma, melanoma, renal cell carcinoma, prostate and breast cancer [179-184]. The efficacy of γδ T cell based immunotherapy has been evaluated in several clinical trials [131, 176]. It was established in these studies that adoptively transferred γδ T cells were well tolerated by the patients and could be safely used as immunotherapy [185].

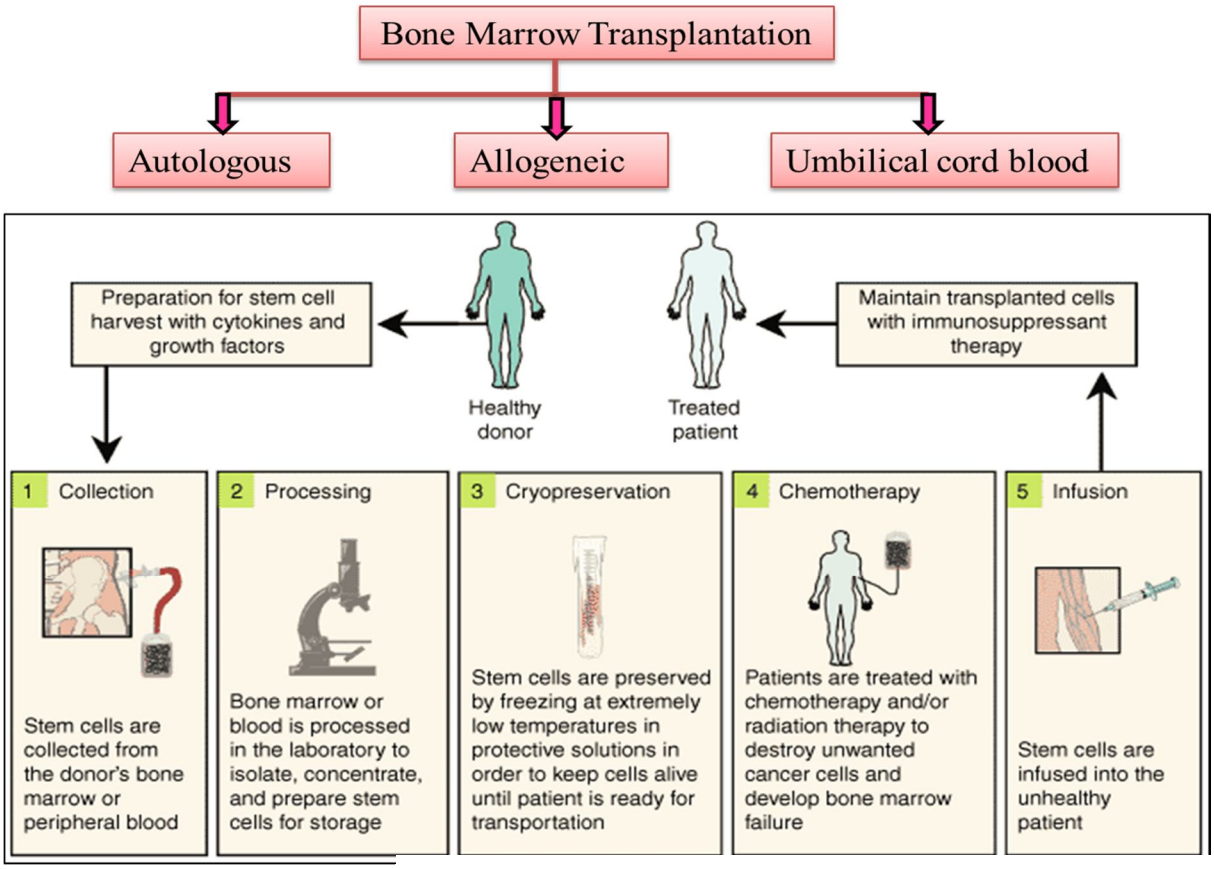


Adapted from Current cancer drug targets 2010;10:27-36

Figure 2.5 Strategies for Vγ9Vδ2 T cell–based immunotherapy of cancer [186].

2.9 Bone marrow transplantation

Bone marrow transplant is a procedure to replace diseased bone marrow with healthy bone marrow stem cells. Bone marrow transplantation is effective in eradicating leukemia in persons with acute and chronic leukemia. There are 3 kinds of bone marrow transplants: 1. Autologous bone marrow transplant: The term auto means self. Stem cells are removed from same patient before high-dose chemotherapy or radiation treatment, are frozen and then readministered. 2. Allogeneic bone marrow transplant: The term allo means other. Stem cells are removed from another person, called a donor. 3. Umbilical cord blood transplant: This is a type of allogeneic transplant. Stem cells are removed from a newborn baby's umbilical cord right after birth. The stem cells are frozen and stored until they are needed for a transplant.



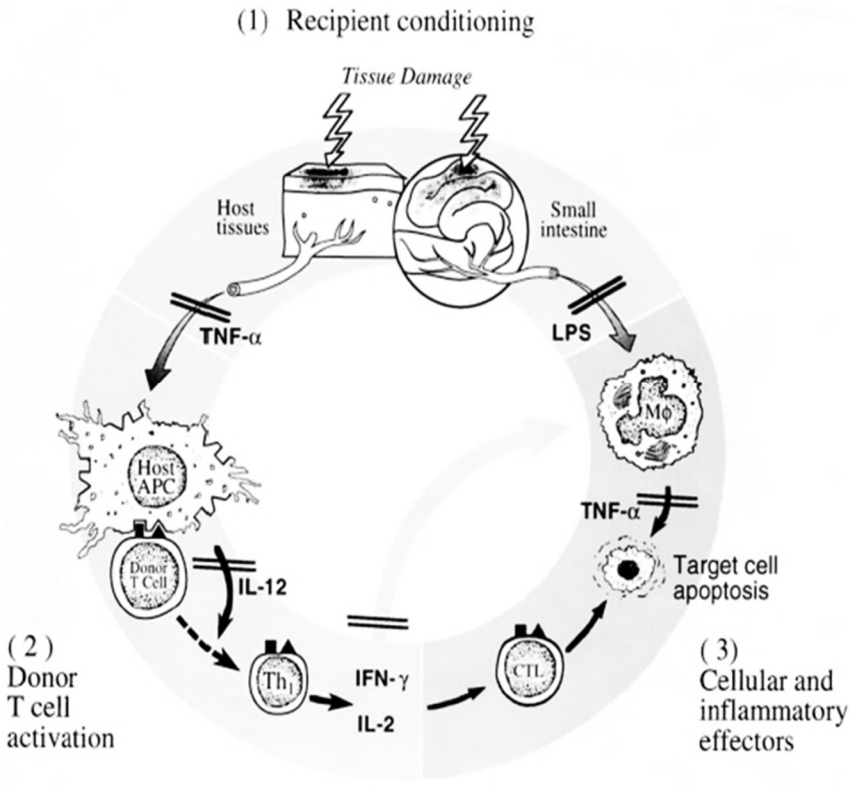
Adapted from Clinical pharmacology and therapeutics 2008;84:620-623

Figure 2.6 Types and procedure of bone marrow transplantation [187]

A transplant with allogeneic haematopoietic stem cells (HSCs) is a treatment in which the transplanted HSCs are obtained from a normal donor. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) can be used to treat either malignant or non-malignant hematologic disorders. Hematopoietic stem cell transplantation from an HLA-haploidentical relative (haplo-HSCT) offers an immediate transplant treatment virtually to any patient in need of an allograft but lacking a suitable matched donor [51]. Mismatches between the histocompatibility antigens of the donor and patient can lead to adverse events, such as rejection of the transplanted graft or pathological immune responses to normal tissues in the patient [188]. In malignant disease, the donor immune system recognizes residual tumor cells as foreign and eradicates them by immunologic means, known as the graft-versus tumor (GVT) effect. Unfortunately, donor immune cells may also attack normal host tissue, particularly the skin, liver, and gastrointestinal (GI) tract, resulting in GVHD [189]. GVHD is

a syndrome in which donor immunocompetent cells recognize and attack host tissues in immunocompromised allogeneic recipients [188].

Acute GvHD occurs most frequently after engraftment, and this has led to an arbitrary period of 100 days post HSCT that has defined the acute versus chronic manifestation of this disease. Clinical manifestations depend on the degree of donor/recipient HLA incompatibility and graft alloreactivity to major host antigens. GvHD can be described as a three-phase process. 1) Afferent phase: In this stage, as is seen with the conditioning of the patient, prior disease and comorbidity of the patient, damage to host tissue occurs. 2) Induction and expansion phase: The second step is the triggering and activation of donor-derived T cells by recipient and donor APC as well as the inflammatory cytokines. 3) Effector phase. Finally, the effector phase is characterized by activated donor T-cell-mediated cytotoxic damage against host cells through Fas–Fas ligand interaction, perforin–granzyme and TNF-α [190].



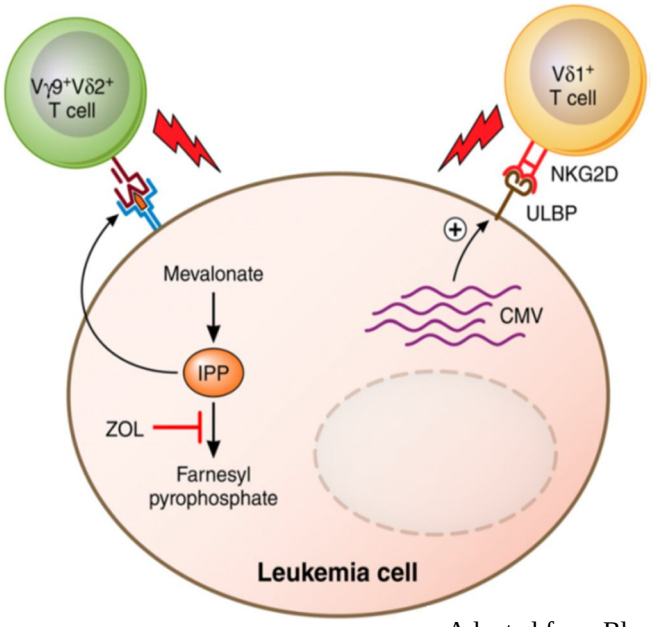
Adapted from JCI 1998;102:115-123

Figure 2.7: Pathogenesis of GvHD [191]

2.10 Role of Gamma Delta T Cells in Haematopoietic Stem Cell Transplantation

γδ T cells combine conventional adaptive features with rapid, innate-like responses that place them in the initiation phase of immune reactions [51]. An increased percentage of total γδ T cells and particularly Vδ2 subsets were demonstrated in patients who developed acute GVHD after transplantation compared to patients without acute GVHD and normal controls, suggesting a possible role of these cells in clinical GVHD [192]. Yabe et al. [193], however, studied 43 allogeneic bone marrow transplant recipients and found that patients with chronic GVHD had a lower proportion as well as absolute number of γδ T cells than patients without chronic GVHD.

Cela et al [194] demonstrated a correlation between increased percentages and number of γδ T cells and the occurrence of viral or fungal infections in patients after HSCT, indicating an active role of γδ T cells in these infections. Lamb et al [195, 196] study confirmed the incidence of higher disease-free survival together with decreased relapse probability in patients with high levels of γδ T cells. Godder et al. [31] also showed highly significant superior 5-year Leukemia free survival and overall survival in patients recovering with increased γδ T cells compared with patients with low/normal γδ T cell percentages. Few studies have reported an active role of γδ T cells in the immune response against CMV in the settings of HSCT [49, 50].



Adapted from Blood 2015;125:2315-2316

Figure 2.8: Putative anti-leukemia and anti-CMV activities of human γδ T cells [197]

Human CMV glycoprotein UL16 binds to the MHC class I–related molecule UL16-binding protein (ULBP) and stimulates the natural killer cell stimulatory receptor NKG2D, also found on Vδ1+ T cells [197].

The antimicrobial effects together with the MHC-independent anti-leukemic potential of γδ T cells suggest a beneficial role in the prevention of GVHD after HSCT. Several factors of potential influence, however, need further clarification in terms of weighing the γδ T cell potential in HSCT; preferably, γδ T cell concentrations and subtypes in the graft and during patient immune reconstitution after HSCT [43]. The potential of combined antiviral and anti-leukemic effects enhances the interest for exploiting adoptive transfer of γδ T cells during early immune reconstruction [49] in the lymphopenic phase after HSCT.

CHAPTER 3

Materials and Methods

3.1 Culture medium

RPMI-1640 medium (Invitrogen Life-Technologies, Grand Island, N.Y.) powder was dissolved in deionized water and supplemented with sodium bicarbonate (Sarabhai Chemicals, India) as per manufacturer's instructions. The medium was sterilized by membrane filtration (0.45mM, Millipore Co, USA), checked for sterility and stored at -20ºC until use.

To prepare complete medium, RPMI plain medium was supplemented with 10% inactivated human AB serum or fetal calf serum, (FCS; Invitrogen Life Technologies, USA), penicillin (100 IU/ml; AlembicChemicals India), streptomycin (100 mg/ml; Alembic chemicals India), gentamycin (40mg/ml; Schering Corpn, India), mycostatin (5 mg/ml;Signa, USA), and L-Glutamine (2mM, Hi Media, India).

3.2 Antibodies

Purified/conjugated antibodies	**Source**
FITC conjugated mouse anti-human TCR Vγ9	BD Pharmingen, USA
PE conjugated mouse anti-human TCR Vδ2	BD Pharmingen, USA
FITC conjugated mouse anti-human TCR Vδ2	BD Pharmingen, USA
FITC conjugated mouse anti-human TCR Vδ1	Thermo Fischer Scientific, USA
PE conjugated mouse anti-human CD3	BD Pharmingen, USA
FITC conjugated mouse anti-human CD3	BD Pharmingen, USA
APC conjugated mouse anti-human CD3	BD Pharmingen, USA

PE-Cy5 conjugated mouse anti-human CD3	BD Pharmingen, USA
PE-Cy7 conjugated mouse anti-human CD3	BD Pharmingen, USA
APC-H7 conjugated mouse anti-human CD3	BD Pharmingen, USA
APC conjugated mouse anti-human CD4	BD Pharmingen, USA
PE conjugated mouse anti-human CD8	BD Pharmingen, USA
PE conjugated mouse anti-human CD19	BD Pharmingen, USA
PE conjugated mouse anti-human TCR γδ	BD Pharmingen, USA
PE conjugated mouse anti-human TCR αβ	BD Pharmingen, USA
PE-Cy7 conjugated mouse anti-human CD25	BD Pharmingen, USA
APC conjugated mouse anti-human CD69	BD Pharmingen, USA
APC conjugated mouse anti-human CD27	BD Pharmingen, USA
PE-Cy5 conjugated mouse anti-human CD45RA	BD Pharmingen, USA
APC conjugated mouse anti-human NKG2D	BD Pharmingen, USA
PE-Cy7 conjugated mouse anti-human CD56	BD Pharmingen, USA
PE conjugated mouse anti-human CD16b	BD Pharmingen, USA
PE conjugated mouse anti-human Perforin	BD Pharmingen, USA
PE conjugated mouse anti-human CD166	BD Pharmingen, USA

PE conjugated mouse anti-human CD6	BD Pharmingen, USA
Mouse anti-human Dynamin-2	BD Pharmingen, USA
FITC conjugated mouse anti-human CD11a	BD Pharmingen, USA
PE conjugated mouse anti-human p-Tyrosine	BD Pharmingen, USA
APC conjugated mouse anti-human HSP60	BD Pharmingen, USA
PE conjugated mouse anti-human HSP70	BD Pharmingen, USA
APC conjugated mouse anti-human MICA	R & D Systems, USA
PE conjugated mouse anti-human MICB	R & D Systems, USA
APC conjugated mouse anti-human ULBP-1	R & D Systems, USA
Mouse anti-human γδ mAb	BD Pharmingen, USA
Mouse anti-human NKG2D mAb	R & D Systems, USA
Secondary antibodies	**Source**
Goat anti-mouse IgG FITC	Sigma Aldrich, USA

3.3 Dyes for cell staining

Dye	**Source**
Cell tracker blue CMAC (7-amino-4-chloromethylcoumarin)	Thermo Fisher Scientific, USA

Phalloidin TRITC (Tetramethylrhodamine B isothiocyanate)	Sigma Aldrich, USA
LysoTrackerRed DND 99	Thermo Fisher Scientific, USA
Calcein AM	Invitrogen, USA

3.4 Recombinant proteins

Protein	**Source**
Recombinant interleukin 2 (rIL2)	Peprotech, USA

3.5 Antigens

Antigen	**Source**
Bromohydrin pyrophosphate (BrHPP/IPH1101)	Innate Pharma, Marseille, France
1-Hydroxy-2-methyl-2-buten-4-yl 4-diphosphate (HDMAPP)	Echelon Bioscience, Salt Lake City, USA
Zoledranate (Zoldonat)	Natco, India

3.6 Study group

Heparinized peripheral blood samples were collected from patients with newly diagnosed T-ALL (n=150), attending the out-patient department at Tata Memorial Hospital (TMH), Mumbai, India. Samples were collected during the period 2011 to 2016. Leukemic blasts in

peripheral blood of these patients ranged from 65-99%. The patients were treated with modified MCP841 chemotherapy protocol as described in table 3.1. 32 patients could be followed up who achieved remission. A complete remission (CR) was defined as less than 5% blasts in a normocellular marrow aspirate and the absence of clinical evidence of disease. Peripheral blood samples were also collected from healthy individuals (n=50). Healthy individuals ranged in the age from 18-30 years. It was not possible to include HI under the age 18 due to ethical issues. Pregnant ladies were excluded. Healthy controls were included if they showed no symptoms of any immediate infections or chronic disease and normal body weight, Hb and if they were not on any long term medications. Informed consent was obtained from all patients and healthy volunteers included in the study. The study was approved by Institutional Ethics Committee of Tata Memorial Centre, Mumbai.

Table 3.1: Modified MCP841 chemotherapy regimen.

Induction	
Tab. Wysolone	40 mg/m2 day 1 to day 29 orally
Inj. Vincristine	1.4 mg/m2 day 1,8,15,22,29
Inj. L-Asparaginase	6000 u/m2 deep I.M. on day 2,4,6,8,10,12,14,16,18,20
Inj. Daunomycin	30 mg/m2 D8, 15, 29
IT MTX	12 mg day 1, 8, 15, 22
Consolidation	
Tab. 6 Mercaptopurine	75 mg/m2 PO day 1 -7, 15-21
Inj. Cyclophosphamide	750 mg/m2 day 1,15
Inj. Cytarabine	2000 mg/m2 I.V. every 12 hours day 1,2;15,16
IT MTX	12 mg day 8, 22
Interim Maintenance	
Tab. 6 Mercaptopurine	75 mg/m2 PO day 1 -7, 15-21
Inj. Cyclophosphamide	750 mg/m2 day 1,15
IT MTX	12 mg day 8, 22
Cranial Radiotherapy	180 cGy/day x 7 #; 10# (for CNS+ pts)
Re-induction	
Tab. Wysolone	40 mg/m2 day 1 to day 29 orally

Inj. Vincristine	1.4 mg/m2 day 1,8,15,22,29
Inj. L-Asparaginase	6000 u/m2 deep I.M. on day 2,4,6,8,10,12,14,16,18,20
Inj. Daunomycin	30 mg/m2 D8, 15, 29
IT MTX	12 mg day 1, 8, 15, 22
Consolidation 2	
Tab. 6 Mercaptopurine	75 mg/m2 PO day 1 -7, 15-21
Inj. Cyclophosphamide	750 mg/m2 day 1
Inj. Vincristine	1.4 mg/m2 day 1,15
Inj. Daunomycin	30 mg/m2 15
Inj. Cytosar	100 mg/m2 S.C. every 12 hourly day 1 – 3; 15-18
IT MTX	12 mg day 1
Maintenance (3 months) x 6 cycles	
Inj. Vincristine	1.4 mg/m2 day 1
Tab. Wysolone	40 mg/m2 day 1 to 7
Inj. L-Asparaginase	6000 u/m2 deep I.M. on day 1, 2, 4, 6
Inj. Daunomycin	30 mg/m2 D 1
IT MTX	12 mg day 1
Tab. 6 Mercaptopurine	75 mg/m2 PO day 15-90
Tab.Methotrexate	15 mg/m2 weekly day 15-90
Abbreviations: Tab, Tablet; Inj, Injection; IT, Intrathecal; MTX, Methotrexate	

3.7 Allogeneic bone marrow transplantation

Patients and their respective BMT donors enrolled at BMT unit, ACTREC, TMC were studied (n=17). 14 patients were diagnosed with B-ALL and 3 were diagnosed with T-ALL. Informed consent was obtained from all patients and healthy donors included in the study. The study was approved by Institutional Ethics Committee of Tata Memorial Centre, Mumbai. Samples were collected during the period 2013 to 2016. The patients were given conditioning chemotherapy with Fludarabine (30mg/m^2 x 5 days) and Melphalan (140mg/m^2 x 1 day) or Cyclophosphamide (60 mg/kg x 2 days) along with total body irradiation (13.2 Gy or 14.4 Gy in 8 fractions). 13 patients received allogeneic hematopoietic stem cell

transplantation (HSCT) from a fully matched related donor (6/6 match), 3 patients received HSCT from haploidentical related donors and 1 patient received HSCT from identical unrelated donor. Human G-CSF-mobilised leukapheresis samples were collected from respective BMT donors. Mobilisation was carried out by daily subcutaneous injection of G-CSF (5µg/kg S.C. BD for 4 days and leukapheresis done on day+5 and day +6). The number of CD34+ cells infused per kg of recipient body weight was 4-5x$10^{6/}$kg. Donor's T-cells were not depleted from grafts. Immune cell phenotype was monitored in recipients at baseline and on day 0, day 30, day 90, day 180 until the one year after HSCT using 3 to 4 mL of peripheral blood. PBMCs were separated using ficoll-hypaque density gradient. Samples were collected weekly for CMV reactivation study till at least day 100 post transplant (analysed using PCR for CMV DNA). Recipients were monitored for various clinical parameters such as engraftment of WBC, engraftment of platelates, development of acute and chronic GvHD, treatment related mortality, and relapse post BMT.

3.8 Separation of peripheral blood lymphocytes (PBLs) and leukemic blasts

Lymphocytes were separated from heparinized venous peripheral blood by Ficoll-Hypaque (FH, Sigma, U.S.A.) density gradient centrifugation. Briefly, peripheral blood collected in heparin (Sigma, USA; 100 IU/ml) was diluted with equal volume of normal saline (0.82% NaCl in double distilled (dd) water). 10 ml of diluted blood was loaded on 2.5 ml of Ficoll-Hypaque [24 parts of 9% Ficoll 400 (Sigma, USA) + 10 parts 33.3% sodium diatrizoate (Sigma, USA), specific gravity adjusted to 1.077 ± 0.001] and centrifuged at 1,500 rpm (200 g) for 20 min at room temperature (RT) using a swing-out rotor in Beckman centrifuge. PBL were collected from the interface between FH and plasma and washed thrice with sterile normal saline. Viability was checked using trypan blue dye and was 100%. Leukemic blasts from peripheral blood of T-ALL patients were also separated using FH density gradient and the viability was >95%.

3.9 Enrichment and purification of γδ T cells from PBLs of healthy individuals

PBL were isolated from healthy individuals (HI) by Ficoll Hypaque density gradient centrifugation and were enriched by stimulation with HDMAPP (1-Hydroxy-2-methyl-2-buten-4-yl 4-diphosphate) (Echelon Bioscience, Salt Lake City, UT) and recombinant IL-2 (rIL-2) as described by Hintz et al. [123].

A. Enrichment of γδ T cells from PBL

$1x10^6$ PBLs were suspended in RPMI 1640 medium containing 10% heat inactivated human AB serum, glutamine (2mM), 2-mercaptoethanol ($5x10^{-5}$M) and antibiotics penicillin (100U/ml), streptomycin (100mg/ml), mycostatin (5mg/ml). rIL2 was supplemented at concentration of (30 IU/ml) in this growth medium. HDMAPP was reconstituted as per manufacturer's instructions and was pre-titrated to decide optimum concentration. PBLs were stimulated with 1 nM/ml HDMAPP in 24 well plate (Nunc, Denmark). Cells were supplemented with rIL-2 every 3rd day and sub-cultured on day 6. On day 12, cells were harvested and γδ T cells were purified using immunomagnetic purification as described by Dhar et al and Gogoi et al [23, 156].

B. Immunomagnetic purification of γδ T cells from expanded PBL

γδ T cells were purified from PBL using TCR-γδ Microbead Kit (Miltenyi Biotech, Germany). Expanded PBLs were washed with MACS buffer (Degassed PBS with 0.5% BSA and 2 mM EDTA). Supernatant was removed and cells were suspended in 40µl of buffer per total 10^7 cells. 10 µl of TCR-γδ hapten mAb was added per 10^7 total cells. Cells were mixed and incubated for 10 min at 4ºC. 30µl of buffer and 20µl of MACS anti FITC micro-beads were added per 10^7 cells, mixed well and incubated for 15 min at 4ºC. Cells were then washed by adding 10-20 times labeling volume of buffer and centrifuged at 1000 rpm for 10 min. Supernatant was removed and cells were suspended in 500 µl buffer per 10^8 cells.

C. Magnetic separation of positively selected cells

MS MACS column (for 10^7 cells) or LS MACS column (for 10^7 to $5x10^7$ cells) was placed in magnetic field of MACS separator and a column was washed thrice with 500µl of wash buffer. 500µl of cell suspension was applied onto the column. Negatively selected cells were washed out from the column and collected separately. Column was removed from the separator and positively selected cells were collected by flushing the cells from the column into 1 ml buffer. Positively selected, FITC labeled cells were checked for the expression of γδ TCR by flow cytometry. Viability of γδ T cells was 100%.

3.10 Immunophenotyping

A. Single/Dual color flow cytometry

A.1. Purity of immunomagnetically purified γδ T cells

Single color flow cytometery was performed on γδ T cells isolated by MACS by acquiring cells on a FACS Calibur flow cytometer (Becton Dickinson). γδ T cells are labeled with anti FITC micro-beads during magnetic labeling and unlabeled cells were kept as isocontrol. Cells were selectively gated for lymphocyte population on a Forward Scatter (FSC) and Side Scatter (SSC) plot. 10,000 events were acquired and data was analysed using FlowJo software (Tree Star, USA). Purity of γδ T cells was > 95%.

A. 2. Detection of immune cell markers

The PBLs were washed and fixed in 1% paraformaldehyde in PBS for 10 min at 4ºC. Cells were washed with PBS. Cells were incubated with mouse anti-human CD3 FITC and CD4 APC or CD8 PE or γδ PE or αβ PE or CD19 PE for 45 min at 4°C. Unstained PBLs were kept as isocontrol and PBLs stained with CD3 FITC or CD3 PE were kept for compensation.

Cells were washed and acquired on the FACS Aria flow cytometer and analysed using FlowJo software (Tree Star, USA).

A.3. Detection of antigens on leukemic blast cells

For analyzing cell surface expression of activation markers, leukemic blast cells from T-ALL patients were left untreated or treated with 100µM Zoledronate for 16-18 hours. Cells were then washed and incubated with anti HSP60 APC, HSP70 PE, MICA APC, MICB PE, ULBP-1 APC, and CD166 PE for 45 min in dark at 4°C. Cells were subsequently washed with FACS buffer (0.01M PBS pH-7.4, 1%FCS, 0.02% Sodium azide), fixed with 1% paraformaldehyde (for longer storage) and the intensity of fluorescence was measured using flow cytometer (FACS Aria, BD Biosciences). Cells were acquired and analysed as mentioned previously.

B. Multicolor flow cytometry

B.1. Surface staining

PBLs from patients and healthy individuals were analysed for T cell subsets (CD3, Vδ1, Vδ2), activation markers (CD25, CD69), memory markers (central memory CD45-CD27+, effector memory CD45-CD27-, naïve CD45+CD27+, TeMRA CD45+CD27-), NK (CD16b, CD56, NKG2D) and NKT (CD3, CD56) cell markers using surface multicolor flow cytometry. PBLs ($1x10^6$) were washed and incubated with different combinations of fluorescently conjugated antibodies. After the incubation at 4°C for 45 minutes in dark, cells were washed with FACS buffer and fixed with 1% paraformaldehyde and the intensity of fluorescence was measured using flow cytometer (FACS Aria, BD Biosciences). Cells were acquired and analysed as mentioned previously.

B.2. Intracellular staining

PBLs ($1x10^6$) were washed and permeabilized with 1% saponin buffer (1% saponin, 0.01M PBS pH-7.4, 1% FCS, 0.02% Sodium azide) for 10 minutes. These cells were incubated with

different combinations of fluorescently conjugated antibodies. PBLs from patients and healthy individuals were analysed for expression of lytic molecules like peforin within CD3, Vδ1 and Vδ2 using intracellular multicolor flow cytometry. After the incubation at 4°C for 45 minutes in dark, cells were washed with FACS buffer and fixed with 1% paraformaldehyde and the intensity of fluorescence was measured using flow cytometer (FACS Aria, BD Biosciences). Cells were acquired and analysed as mentioned previously.

3.11 Immune synapse formation between γδ T cells and leukemic targets

A) Labeling of cells

A.1. Labeling with CMAC blue

CMAC blue was reconstituted as per manufacturer's instructions. CMAC blue was pre-titrated and the best concentration was used for the assays. γδ T cells ($1x10^6$) were washed and 100 µl CMAC (5µM) was added to cell pellet. Cells were incubated for 30 min., at 37°C and then washed twice with medium. These CMAC blue labeled cells were used further for co-culture assay.

A.2. Labeling with LysoTracker Red (LTR)

LTR was pre-titrated and the best concentration was used for the assays. γδ T cells ($1x10^6$) were washed and 100 µl LTR (100nM) was added to cell pellet. Cells were incubated for 30 min., at 37°C and then washed twice with medium. These LTR labeled cells were used further for co-culture assay.

A.2. Labeling with Calcein AM

Leukemic blast cells ($0.5x10^6$) were suspended in 1 ml plain RPMI medium. 2 µl Calcein (1mM) was added to cell suspension and incubated at 37°C for 15 min. and then washed twice with medium. These Calcein AM labeled cells were used further for co-culture.

B) Time-lapse confocal microscopy

Leukemic blasts ($0.5x10^6$) from patients were kept untreated or treated with 100 µM Zoledronate for 16-18 hours and then washed with plain RPMI. Purified γδ T lymphocytes were labeled with LysoTracker red (50nM), to visualize the acidic perforin granules and Cell Tracker Blue CMAC (5µM), as described previously. Labeled γδ T cells were mixed with Calcein AM (1mM) labeled leukemic blasts and centrifuged for 2 minutes at 500 rpm. These conjugated cells were co-incubated for 30 min. at 37^0C. These cell conjugates were then transferred to 35 mm glass bottom petri dish (Cell E&G, Houston, TX, USA). Conjugates were examined using a Zeiss 780 Meta confocal laser-scanning microscope (Carl Zeiss, Jena, Germany) using a X63 objective lens. Images were acquired every 1 min. for 30-45 min. Images were processed using image processing software Zen (Zeiss, Germany).

B) f-Actin polarization

Leukemic blasts ($0.5x10^6$) from patients were kept untreated or treated with 100 µM Zoledronate for 16-18 hours and then washed with plain RPMI. Purified γδ T lymphocytes were labeled with Cell Tracker Blue CMAC (5µM). Labeled γδ T cells were mixed with Calcein AM (1mM) labeled leukemic blasts. These conjugates were fixed with 1% paraformaldehyde and permeabilized with 5% Triton X100 and thereafter labeled with 40 µl working stock of Phalloidin TRITC for 15 min. at 37^0C.

Working stock of Phalloidin TRITC is prepared as below:

Component	**Volume**
Lysolecithin (10µg/ µl)	2 µl
Methanol (AR Grade)	20 µl
1X PBS (pH 7.4, 0.01 M)	171 µl

BSA (2%)	5 µl
Phalloidin TRITC (2 mg/ml)	2 µl

Conjugates were incubated with 2% BSA for 10 min. at RT and washed with 1X PBS. These cell conjugates were then transferred to 35 mm glass bottom petri dish and f-Actin polarization was examined using a Zeiss 780 Meta confocal laser-scanning microscope using a X63 objective lens. Images were acquired and analysed as described previously. Entire petri dish was scanned and minimum 10 conjugates were analysed per plate.

B) Analysis of molecules involved in immune synapse formation

B.1. CD11a and Dynamin-2 expression

Purified γδ T lymphocytes were co-cultured with CMAC blue (5µM) labeled untreated or Zoledronate treated (100 µM) leukemic blasts for 30 min. at 37^0C. These conjugates were fixed with 1% paraformaldehyde and then incubated with FITC conjugated CD11a (LFA-1) or purified mouse anti human Dynamin-2 for 30 min. at 4^0C. Secondary goat anti mouse FITC antibody was added against Dynamin-2 labeled conjugate. These conjugates were washed and acquired as described previously.

B.1. CD166 and p-Tyrosine expression

Purified γδ T lymphocytes were labeled with Cell Tracker Blue CMAC (5µM) following the manufacturer's instructions. Labeled γδ T cells were co-cultured with Calcein AM (1mM) labeled untreated or Zoledronate treated (100 µM) leukemic blasts for 30 min. at 37^0C. These conjugates were fixed with 1% paraformaldehyde and permeabilized with 1% saponin buffer. Conjugates were incubated with PE conjugated CD166 or PE conjugated p-Tyrosine for 30 min. at 4^0C. These conjugates were washed and acquired as described previously.

3.12 Analysis of clonality in T-ALL patients

A) DNA extraction

DNA was extracted from leukemic blasts using a commercially available Qiagen DNeasy kit (Hilden, Germany). The density of the cells was adjusted to 5-10x10^6 cells and incubated in 200µl of 1X PBS, 20µl proteinase K and 4µl RNaseA (1mg/ml). Cells were incubated at 56ºC after adding the lysis buffer provided in the kit. Cells were transferred onto a DNeasy minispin column after addition of ethanol and centrifuged at 8000 rpm for 5 min. The column was washed twice and the DNA eluted using elution buffer. The concentration and purity of DNA was determined at an optical density ratio of 260/280 using the Nanodrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and Quality of extracted DNA was checked by 0.8% agarose gel electrophoresis.

B) γδ PCR

PCR amplification and heteroduplex analysis of TCR γ and δ gene rearrangements was performed as described earlier [95, 198]. The primers were obtained as gift from Dr. van Dongen, (Erasmus University, Rotterdam, The Netherlands) and the sequences are published [63].

List of primers used for γδ PCR

Direction	Primers	molecular weight	A260	pM/µl
5'	VγI	6417.2	0.345	887
5'	VγII	6456.2	0.266	680
5'	VγIII	7341.8	0.476	1070
5'	VγIV	7583	0.732	800
3'	Jγ1.1	7386.8	0.745	832
3'	Jγ1.2	7992.2	0.423	873

3'	Jγ1.3	6983.6	0.455	1075
5'	Vδ1	7239.8	0.501	1140
5'	Vδ2	7670	0.428	921
5'	Vδ3	6528.2	0.442	1120
5'	Dδ1	8254.4	0.447	893
3'	Jδ1	6276.2	0.36	946

The reaction mixture for PCR in 50 µl contained 200ng template DNA, 6.25 pmoles of 5' and 3' oligonucleotide primers, 1.25mM dNTP, 20mM Tris-HC1 pH 8.3, 1.5 mM MgCl2 and 0.5u Taq polymerase and the products were checked on 2% agarose gel after ethidium bromide staining.

PCR cycles:

Step	Temperature(°C)	Time (min)
1	94	3
2	94	1
3	60	1
4	72	2
5	Go to step 2, 34 times	
6	72	7
7	4	Soak

Base pair sizes of the PCR products:

Sr.no.	Rearrangement	Base pair size
1	VγI- Jγ1.1	352
2	VγI- Jγ1.2	350
3	VγI- Jγ1.3	554
4	VγII- Jγ1.1	341
5	VγII- Jγ1.2	339
6	VγII- Jγ1.3	543
7	VγIII- Jγ1.1	330
8	VγIII- Jγ1.2	328
9	VγIII- Jγ1.3	532
10	VγIV- Jγ1.1	369
11	VγIV- Jγ1.2	367
12	VγIV- Jγ1.3	571
13	Vδ1- Jδ1	472
14	Vδ2- Jδ1	460
15	Vδ3- Jδ1	456
16	Dδ1- Jδ1	715

C) Heteroduplex analysis

In heteroduplex analysis, homo- and heteroduplexes resulting from denaturation (at 94°C) and renaturation (at lower temperatures) of PCR products are separated in non-denaturing polyacrylamide gels based on their conformation.

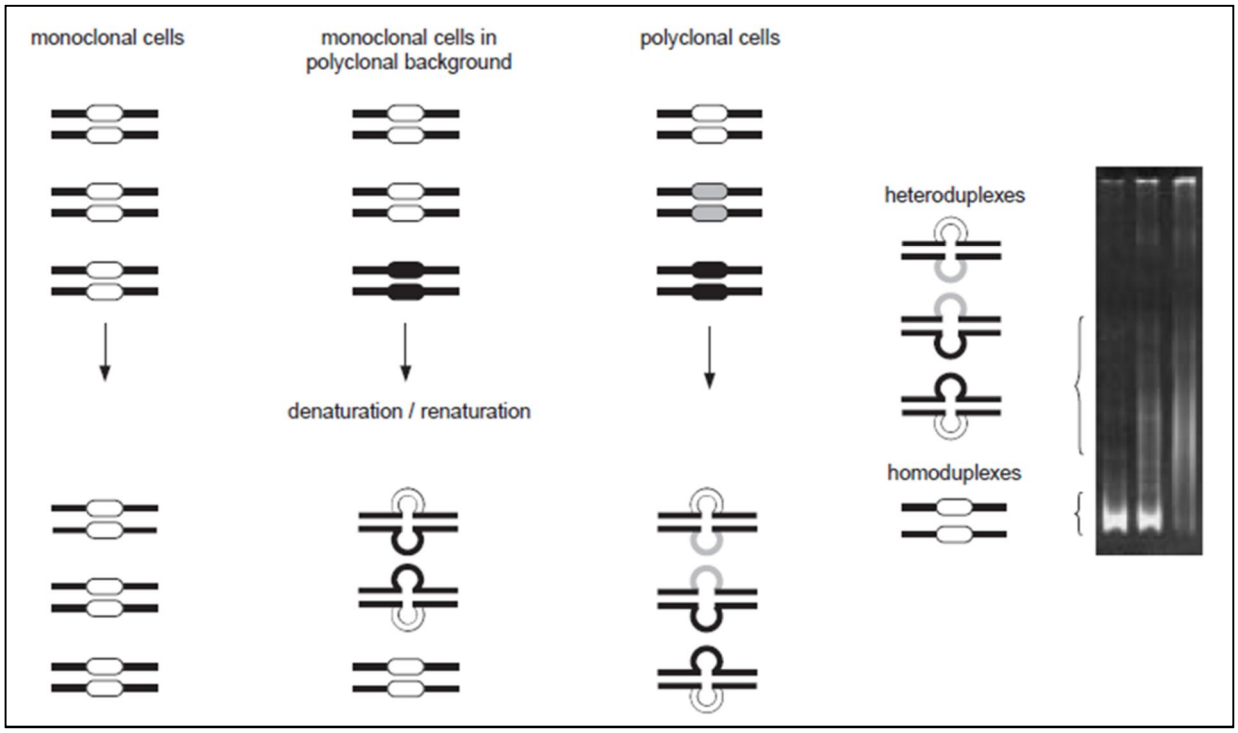


Figure 3.1: Schematic diagram of the heteroduplex analysis technique.

Homoduplexes with perfectly matching junctional regions migrate more rapidly through the gel than heteroduplex molecules with less perfectly matching junctional regions. The latter form a background smear of slower migrating fragments. The presence of clear homoduplex bands or a smear of heteroduplexes enables discrimination between monoclonality and polyclonality, respectively [198].

For heteroduplex analysis, 20µl of each amplified PCR product was heated at 94°C for 5 minutes and rapidly cooled to 0°C and left at this temperature for 1 hr in a thermal cycler. The samples were run on 6% non-denaturing polyacrylamide gel (29: 1, acrylamide: bisacrylamide) in Tris-borate-EDTA buffer. The gel apparatus was assembled. The plates were sealed and the gel was allowed to polymerize and the assembly was set inside the electrophoresis tank (LKB, Produkter, Sweden).The samples were loaded with the help of Hamilton syringe and were run at constant voltage of 150V for appox 2.5 hrs.

Reagents for preparation of 6% PAGE gel:

Reagents for 6% PAGE	Volume (ml)
10X Tris Borate EDTA	2
29:1 Acrylamide: Bisacryamide	8
10% Ammonium persulphate	240 µl
TEMED	60 µl
Milli Q water	30

Preparation of the gel loading sample:

Reagent	Volume
Product/ sample	20µl product + 5 µl Bromophenol blue
Marker	1µl marker + 19µl 0.5X TBE + 5µl BPB

Heteroduplex patterns were studied by silver staining method.

3.13 cDNA microarray

A) RNA Extraction and RNA Quality Control

Total RNA was isolated using TRIZOL Reagent following the standard protocol (Invitrogen, Carlsbad, CA). RNA concentration and purity was determined at an optical density ratio of 260/280 using the Nanodrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the integrity of total RNA was verified on an Agilent 2100 Bioanalyzer using the RNA 6000 Nano LabChip (Agilent, Palo Alto, CA) following the manufacturer's protocol. RNA was used further for microarray experiments if RNA Integrity Number (RIN) was =7.0.

B) Labeling

The microarray hybridization and scanning were performed at the Agilent certified microarray facility of Genotypic Technology, Bengaluru, India. The samples for Gene expressions were labeled using Agilent Quick Amp Kit PLUS (Part number: 5190-0442). Five hundred nanograms each of the samples were incubated with reverse transcription mix at 42°C and converted to double stranded cDNA primed by oligodT with a T7 polymerase promoter. The cleaned up double stranded cDNA were used as template for cRNA generation. cRNA was generated by in vitro transcription and the dye Cy3 CTP(Agilent) was incorporated during this step. The cDNA synthesis and in vitro transcription steps were carried out at 40°C. Labeled cRNA was cleaned up and quality assessed for yields and specific activity.

C) Hybridization, scanning and Feature Extraction

The labeled cRNA samples were hybridized on to a Whole Genome Human Array 4x44k (AMADID: 14850). 1650 ng of cy3 labeled samples were fragmented and hybridized. Fragmentation of labeled cRNA and hybridization were done using the Gene Expression Hybridization kit of Agilent (Part Number 5188-5242). Hybridization was carried out in Agilent's Surehyb Chambers at 65º C for 16 hours. The hybridized slides were washed using Agilent Gene Expression wash buffers (Part No: 5188-5327) and scanned using the Agilent Microarray Scanner G Model G2565BA at 5 micron resolution. Data extraction from Images was done using Agilent Feature Extraction software (v9.5).

E) Microarray Data Analysis

Feature extracted data was analysed using Agilent GeneSpring GX version 7.3.1 Software (Agilent Technologies, Inc., Santa Clara, CA, USA) and TM4 Microarray Multiexperiment Viewer (T_MeV) (Boston, MA, USA). Normalization of the data was done in GeneSpring GX using the recommended one color Per Chip and Per Gene Data Transformation: Set

measurements less than 0.01 to 0.01, PerChip: Normalize to 50th percentile, Per Gene: Normalize to Specific Samples and median of all samples. Significant genes high and low expressed among the groups were identified with cutoff 2 fold difference. Fold change was calculated as log base 2. Statistical p-value was calculated using student T-test and volcano Plot. Significance Analysis of Microarray was also performed to identify differentially regulated genes. Differentially regulated genes were clustered using hierarchical clustering. Significant genes were clustered using gene tree to identify significant gene expression patterns. Genes were classified based on functions and pathways using biological interpretation tool Biointerpreter (Genotypic Technology) and GeneSpring Software.

F) Functional analysis by gene ontology

Differentially regulated genes between the two subgroups were analysed for gene ontology (GO) annotations using DAVID Bioinformatics Resources 6.7 (National Institute of Allergy and Infectious Diseases, NIH) [199, 200]. We also used the Biointerpreter software (http://www.genotypic.co.in/biointerpreter) for gene ontology analysis. Differentially expressed genes from normalized data set were subjected to functional annotation and statistically significant genes were categorized into Biological Process, Molecular Function and Cellular Components. Functional annotation was done using gene set enrichment analysis describing EASE score (One tail Fisher exact probability value). The gene expression data is deposited in Gene Expression Omnibus (GEO) Database with an accession no.GSE37389.

3.14 Quantitative Real Time PCR (qRT-PCR) For Validation of Gene Expression

A. Extraction of RNA

Leukemic blasts from T-ALL patients were stored at –80ºC in TRIzol (Invitrogen Life-Technologies, N.Y.) in a ratio of 1X10^6 cells/100 µl TRIzol solution until further use. At the

time of RNA extraction, 0.1 volume (1/10th of Trizol) 24:1 CHISAM (Chloroform : Isoamyl alcohol) was added, vortexed for 15 seconds and incubated on ice for 15 minutes. This solution was centrifuged at 14,000 rpm for 10 min and the aqueous phase was collected. Equal volume (equal to aqueous phase) of chilled isopropyl alcohol (Qualigens, India) was added to aqueous phase and mixed gently and incubated at -80^{0}C for 2 hours and then centrifuged at 14,000 rpm for 10 min. After centrifuging, the pellet obtained was washed with 80% ethanol (14,000 rpm for 10 min). The pellet was again washed with 100% ethanol (14,000 rpm for 10 min). The pellet was air dried and dissolved in appropriate volume of DEPC (Sigma)-treated water. Optical density (O.D.) readings were taken for quantitation of RNA by NanoDrop spectrophotometer (Thermo Scientific, DE). The RNA was run on a 2% agarose gel containing ethidium bromide to confirm its purity and integrity.

B. Complementary DNA (cDNA) synthesis by reverse transcription

Total RNA from the blast cells was used for first strand cDNA synthesis using oligo dT primers (Invitrogen Life-Technologies, N.Y.). 5µg of RNA (10 µl volume with DEPC treated water) was reverse transcribed using 1µl of oligo dT and 1µl (10 mM) of dNTP; this mixture was heated at 65ºC for 10 min and then cooled at 4ºC for 10 min. The mixture for reverse transcriptase containing the components given below was prepared and was added to the previously prepared RNA-primer mixture.

Component	**Volume**
5X 1st strand buffer	4 µl
0.1 M DTT	2 µl
RNase inhibitor	1 µl

This total mixture was heated at 37ºC for 2 min and then reverse transcribed using 1µl reverse transcriptase enzyme (2000U/µl, Murine Moloney Leukemia Virus reverse transcriptase enzyme, Invitrogen Life-Technologies, N.Y.) at 37ºC for 50 min in PTC-100™ Programmable Thermal Controller (MJ Research Inc.). The mixture was heated at 70ºC for 15 min. The final cDNA volume was 20µl.

C. qRT-PCR

Quantitative RT-PCR for different genes shortlisted from microarray analysis (NFkB, FASLG, HLA-DQB, IFNB1, RXRA, CISH, SOCS2) carried out on ABI Prism 7900HT sequence detection system (PE Applied Biosystems, Foster City, CA). For qRT-PCR, Taq-Man Fast Universal PCR Master Mix and Assay-on-demand TaqMan Gene Expression Assay primer/probe mixes (Applied Biosystems, Foster City, CA, USA) were used.

RT-PCR reaction mixture was prepared:

Component	**Volume**
100ng cDNA + DEPC water	2.25 µl
20X Primer probe mixture	0.25µl
2X Master-mix	2.5µl

The primer/probes used for quantitative real time PCR were: NFkB (Hs00765730_m1), FASLG (Hs00899442_m1), HLA-DQB (Hs03054971_m1), IFNB1 (Hs00277188_S1), RXRA (Hs01067635_m1), CISH (Hs00367082_g1), SOCS2 (Hs00374416_m1). Relative mRNA level expression was quantitated using β-Actin (*ACTB*; NM_001101) as

housekeeping gene. Fold changes in target gene expression were normalized to housekeeping gene via the published comparative $2^{-\Delta\Delta Ct}$ method [201].

3.15 Droplet digital PCR

Droplet Digital PCR (ddPCR) was carried out using the QX200 Droplet Digital PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Samples were analysed for mutations in *NOTCH1* (pL1600P), *IKZF3* (pL162R) and *BRAF* (pV600E) genes and Copy Number Variations in *P53, CDKN2A* and *CDKN2B* genes. ddPCR reactions were of 20 µl aqueous volumes that contained final concentrations of 1X ddPCR supermix (Bio-Rad), 0.3 µM each primer and probe and 20 to 50 ng of genomic DNA. Annealing temperature for mutation detection primers was calibrated using gradient PCR and found to be 55°C. Amplifications were performed using the following conditions: For mutation detection: 1 cycle of 95°C for 10 min, 40 cycles of 94°C for 30 s and 55°C for 1 min, and 1 cycle of 98°C for 10 min and for CNV: 1 cycle of 95°C for 10 min, 40 cycles of 94°C for 30 s and 60°C for 1 min, and 1 cycle of 98°C for 10 min. Droplet generation and droplet reading for ddPCR were carried out according to the manufacturer's instructions using Bio-Rad reagents. *RPP30* was used as reference gene in CNV analysis. Results were analysed using QuantaSoft Software (Bio-Rad) and represented as copy numbers and concentration of mutation (copies/µl).

3.16 Cytotoxicity assay

Cytotoxic potential of γδ T lymphocytes (used as effectors) was studied against leukemic blast cells (used as a target) using ^{51}Cr Release Assay. Leukemic blasts from T-ALL patients were either treated with Zoledranate (100 µM) or kept untreated for 16-18 hours at 37˚C. These untreated/ treated blasts were washed with pRPMI and used for labeling. Leukemic blasts (1 x 10^6) were labeled with 50 µCi of ^{51}Cr (sodium chromate, Amersham, UK) for 1 hour at 37˚C. Purified γδ T cells were titrated at various effector: target (γδ: leukemic blasts) ratios (40:1, 20:1, 10:1, 5:1 and 2.5:1) in triplicates and added to ^{51}Cr-labeled leukemic blasts

(5 x 10^3). Leukemic blasts were co-cultured with γδ T lymphocytes for 4 hours at 37°C in 96-well plates (Nunc, Denmark). After 4 hours, plates were centrifuged, supernatants were collected and the released radioactive chromium was measured using 1470 Wallac automated gamma counter (Perkin-Elmer, Downers Grove) and expressed as counts per minute (cpm). The maximum release of chromium was determined by lysing leukemic blasts with Triton X-100 (5%) while the spontaneous release was determined by incubating only leukemic blasts in medium for 4 hours. The % cytotoxicity of γδ T cells was calculated as follows: % cytotoxicity = [(mean experimental CPM - mean spontaneous CPM)/ (mean maximum CPM - mean spontaneous CPM)] x 100.

3.17 Cytotoxicity inhibition assay

Role of γδ TCR and NKG2D receptor in the killing of leukemic blast was studied using cytotoxicity inhibition assay. γδ T cells were incubated with anti-pan γδ mAb (10 µg/ml, clone B1, BD Pharmingen, USA), anti-NKG2D mAb (20 µg/ml, R&D Systems, USA) or a combination of TCR and NKG2D antibodies for 1 hour at 37˚C before adding to the targets at an E:T ratio of 40:1. 1 x 10^6 leukemic blast cells (untreated/ Zoledronate treated) were labeled with 100 µCi of ^{51}Cr (sodium chromate, Amersham, UK). Isotype IgG control antibody was used as a negative control. The % inhibition of cytotoxicity was determined as follows: % inhibition of cytotoxicity = 1-(cytotoxicity in the presence of the mAb/cytotoxicity in the absence of the mAb) x100.

3.18 Co-culture assay

Co-culture experiment was performed to study the effect of TCRαβ and TCRγδ clonal leukemic blasts on healthy PBMCs. Leukemic blasts (0.1x10^6) from T-ALL patients were co-cultured with healthy PBMCs in two ratios, 1:10 (0.1x10^6 blasts: 1 x10^6 PBMCs) and 1:30 (0.1x10^6 blasts: 3 x10^6 PBMCs) for 12 days with or without addition of rIL-2 on day 0 in RPMI supplemented with 10% AB serum. rIL-2 (30 IU) was added to both the sets on every

3rd day and cells were sub-cultured on day 6. On day 12, culture was harvested and dual color immunophenotyping was carried out for various cell surface markers, like CD3, Vδ2, αβ T cells.

3.19 CFSE assay

CFSE assay was performed to study the proliferation of healthy PBMCs in response to TCRαβ or TCRγδ clonal leukemic blasts. CFSE labeling was performed according to Quah et al [202]. In brief, $10x10^6$ PBMCs were suspended in CFSE buffer (1X PBS + 5% FBS) and added to bottom of 15 ml sterile tube. 1.1 µl CFSE (5mM, Molecular Probes, Eugene, USA) was added to 110 µl CFSE buffer to get 5 µM CFSE. Cell suspension was mixed and vortexed with CFSE and incubated in dark for 5 min. After the incubation, labeled cells were washed twice with CFSE buffer and were used for assay. For the assay, $0.1X10^6$ leukemic blasts were co-cultured with healthy PBMCs, which were previously labeled with CFSE in 1:10 ratio for 6 days in presence of BrHPP (200nM) and rIL2 (30IU). PBMCs with rIL-2 (30IU) and BrHPP (200nM) were kept as positive control and PBMCs with rIL-2 (30IU) alone were kept as negative (baseline) control. On day 6, culture was harvested and dual color immunophenotyping was carried out using CFSE/Vδ2 PE and CFSE/αβ PE. Cells were acquired using FACS Aria flow cytometer and proliferation of Vδ2 and αβ T cells was analysed using FlowJo software.

3.20 Statistical analysis

T-ALL patients recruited in the study were followed up after start of treatment till end of study. Overall survival (OS) was calculated from the date of commencement of treatment to the date of last follow-up or death. Disease free survival (DFS) was calculated from date of complete remission to the first event/relapse or death or last follows up. Patients receiving allogeneic BMT were also monitored post BMT till one year.

Overall survival (OS) and disease free survival (DFS) were evaluated according to Kaplan–Meier survival analysis using SPSS Graduate Pack 21.0 software (SPSS Inc, Chicago, IL, USA). Graphs were plotted using Prism 5 software (GraphPad Software, Inc, CA, USA). P values were assessed using log-rank test. $P<0.05$ was considered as statistically significant. In BMT patients, Cox regression analysis was performed to study the correlation of different clinical and immune variables with clinical outcome. P value was calculated using Fischer exact test (2 sided). Immune cell numbers were grouped in high or low according to median value. The effect of immune cell phenotype on CMV reactivation was analysed using Kaplan Maier failure analysis in Stata software (Texas, USA).

CHAPTER 4

Result-I

Immune scenario in T-ALL patients and the functional role of γδ T cells against leukemic blasts

Patients with T-ALL tend to present with very high circulating blast cell counts, mediastinal masses, and central nervous system involvement. The prognosis of T-ALL in children and adolescents has improved in recent years due to intensified therapies, with 5 year relapse-free survival rates now in the range of 60%–75% [58, 59]. Immunophenotyping of leukemic lymphoblasts by flow cytometry is essential to establish the correct diagnosis and define cell lineage. Acute lymphoblastic leukemia can be readily sub-classified according to the many steps of normal B-cell and T-cell differentiation and phenotypes [3]. γδ T cells exhibit potent MHC-unrestricted lytic activity against several tumor cells. γδ T cell-based immunotherapy against different tumors has been reported [203-206], and the efficacy of this treatment has been demonstrated both in vivo and in vitro [139, 181, 183, 207].

In the present chapter T-ALL patients were studied for their immune cell composition at different stages of disease viz. at diagnosis and at remission and it was compared with healthy individuals. The ability of γδ T cells to lyse T-ALL patient's blast cells was studied and the molecules involved in conjugate formation with leukemic blasts were investigated.

4.1 Study group

Study was performed on 150 T-ALL patient samples, accrued from 2011 to 2016. Characteristics of patients diagnosed with T-ALL were obtained from Tata Memorial Hospital EMR (Electronic Medical Records). Patients were treated with modified MCP-841 chemotherapy protocol as mentioned in chapter 3, table 3.1. A general male predominance was observed in patients accrued, with a total of 121 (80.66 %) males and 29 (19.33 %) females. The lymphocyte count was observed to be very high in almost all the patients with median absolute lymphocyte count of 116.42 x 10^6/ml ranging from 1.2 x 10^6/ml to 420 x 10^6/ml. Patient's age ranged from 11 months to 30 years (median: 9 years). Majority of patients (134 patients, 89.33%) were less than 15 years old. Blast count was high in all the patients with a median of 90% (42 to 99%). 32 T-ALL patients achieving remission (blast count < 5%, Hb and WBC count in normal range and no symptoms of disease) could be followed up and were included in the study.

4.2 Immunophenotyping of T-ALL patients at diagnosis and remission

Immunophenotype of the T-ALL patients at diagnosis (n=116) and remission (n=32) and healthy individuals (n=15) was carried out. Multiparametric flow cytometry was used to study multiple surface as well as intracellular markers simultaneously such as, γδ T cell subsets (Vδ1, Vδ2), activation markers (CD25, CD69) and Memory markers (central memory $CD45^-CD27^+$, effector memory $CD45^-CD27^-$, naïve $CD45^+CD27^+$, TeMRA $CD45^+CD27^-$), CD4, CD8, CD56, NKG2D, CD16b, and Perforin. PBMCs from T-ALL patients were stained with fluorescently tagged antibodies and 10,000 events were acquired on FACS Aria using FACS Diva software. In dual and multicolour staining, post-acquisition compensation was performed and only cells (PBMCs) were used as an isocontrol (unstained negative control for selecting the population for analysis). Acquired data was analysed using FlowJo software. The multiple gating strategies performed are represented in figure 4.1.

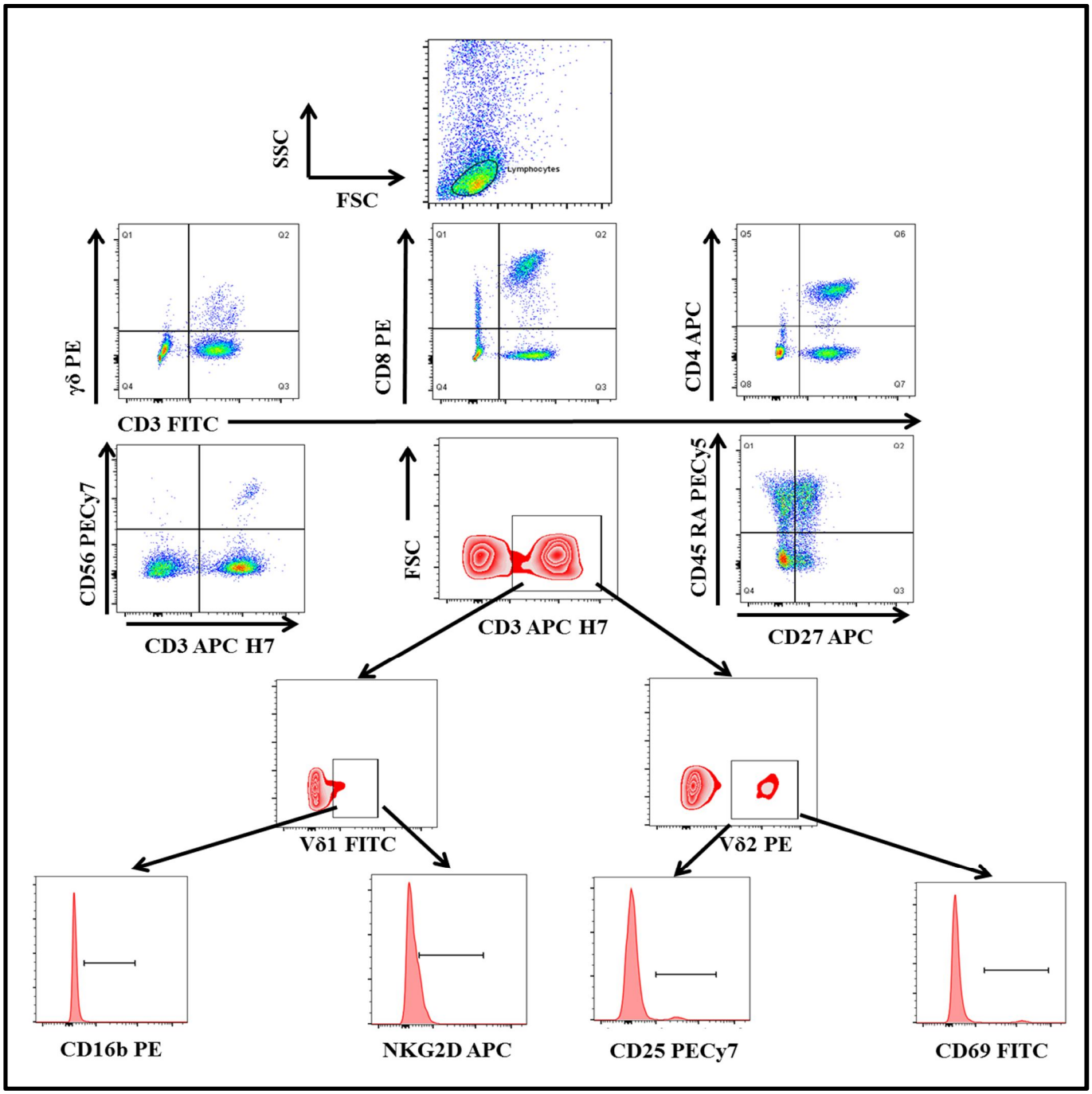


Figure 4.1 Immune cell markers studied using multicolour flow cytometry. A representative figure showing the gating strategies used to analyse multiple surface and intracellular immune cell markers in T-ALL patient samples. Lymphocytes were selected using forward versus side scatter. CD3, Vδ2 and Vδ1 cells were gated in lymphocyte population. Histograms and dot plots were plotted to analyse multiple immune cell markers within these cell populations. Graphs were plotted using FlowJo software.

a) Immune cell subsets

T-ALL patients at diagnosis showed significantly reduced numbers of total CD3, Th (CD4), Tc (CD8), NK, and αβ T cells as compared to HI as shown in fig.4.2. However the levels of NKT cells were comparable to those observed in HI. It was interesting to note that T-ALL patients in remission showed significantly elevated levels of CD3, CD4 (Th), CD8 (Tc), NKT, NK, and αβ T cells compared to patients at diagnosis indicating a marked improvement in their immune status. Although the immune cell numbers improved significantly in patients achieving clinical remission, these were not comparable to HI. The results indicate that T-ALL patients at diagnosis have low numbers of immune cells in peripheral blood which may lead to decreased immune functions. Every figure represents data of n=116 (at diagnosis), n=32 (at remission) and n=15 (HI).

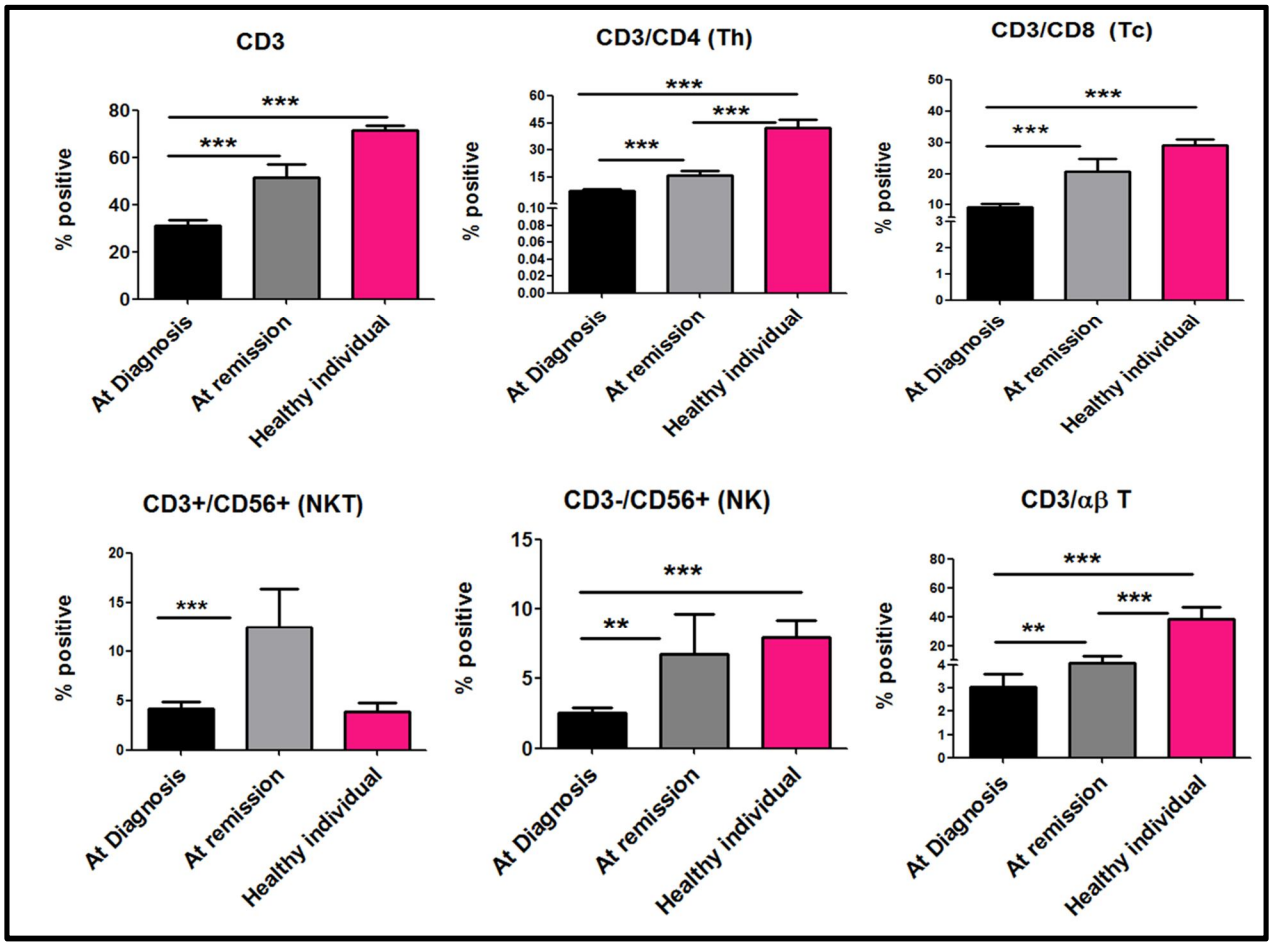


Figure 4.2 Immunophenotyping in T-ALL patients compared to HI. Figure represents % positive expression of immune cell markers (CD3, CD4, CD8, NKT, NK and αβ T cells) in T-ALL patients at diagnosis (black bar), at remission (grey bar) compared to healthy individuals (pink bar). Data is represented as mean±SE, *: p<0.05, **:p<0.005, ***:p<0.0005

b) γδ T cell subsets

γδ T cells and their subsets were studied in T-ALL patients at diagnosis, at remission and HI. Patients at diagnosis showed significantly reduced numbers of γδ T cells, and Vδ2 and Vδ1 T cells, the two major γδ T cell subsets, compared to healthy individuals. T-ALL patients at remission showed significant increase in the numbers of γδ T cells, Vδ2 and Vδ1 T cells compared to patients at diagnosis. The levels of γδ T cells and its subsets in remission patients were comparable to HI.

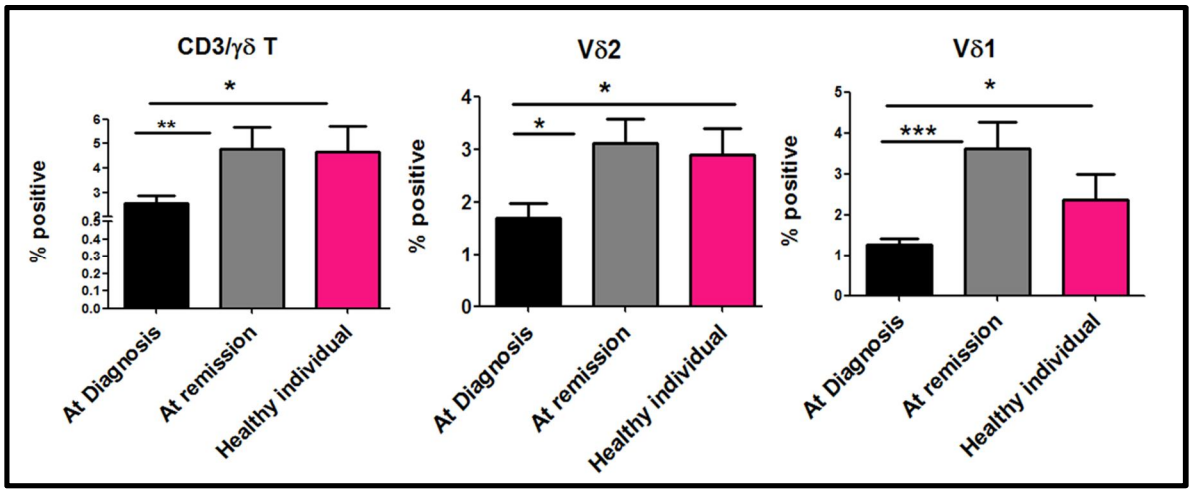


Figure 4.3 Expression of γδ T cell subsets in T-ALL patients compared to HI. Figure represents % positive expression of γδ T cells, Vδ2 and Vδ1 T cells in T-ALL patients at diagnosis (black bar), at remission (grey bar) as compared to healthy individuals (pink bar). Data is represented as mean±SE, *: p<0.05, **:p<0.005, ***:p<0.0005

c) Activation markers

CD3, Vδ2 and Vδ1 T cells were studied for their early and late activation status (CD69 and CD25 respectively) in T-ALL patients at diagnois, at remission and HI. T-ALL patients at diagnosis showed comparable levels of early and late activation

markers (CD69 and CD25 respectively) compared to HI. Patients in remission showed significantly higher expression of late activation marker (CD25) on CD3, Vδ2 and Vδ1 T cell subsets, as compared to patients at diagnosis as well as healthy individuals as shown in figure 4.4. The levels of early activation marker on Vδ1 T cells were significantly higher in remission patients as compared to HI, whereas there was no significant difference in early activation of CD3 and Vδ2 T cells.

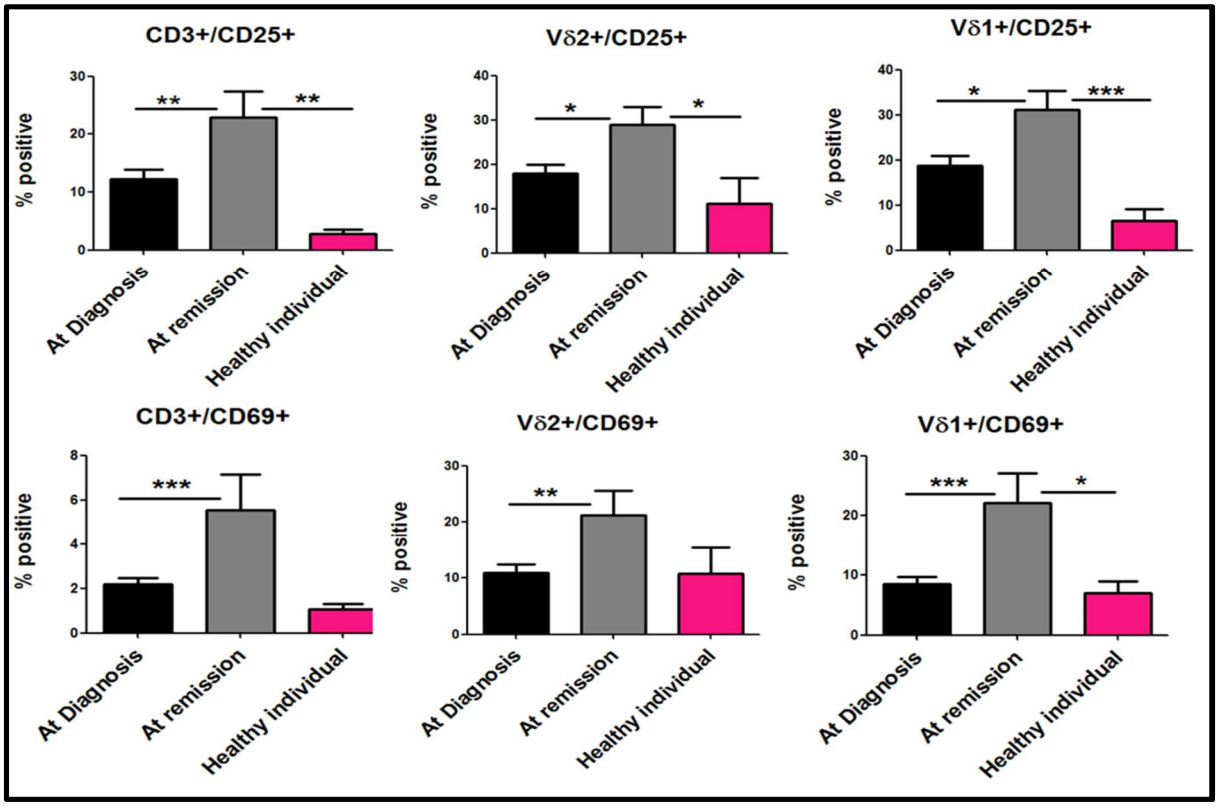


Figure 4.4 Expression of activation markers on CD3, Vδ2 and Vδ1 T cell subsets in T-ALL patients compared to HI. Figure represents % positive expression of early and late activation markers (CD69 and CD25 respectively) in T-ALL patients at diagnosis (black bar), at remission (grey bar) as compared to healthy individuals (pink bar). Data is represented as mean±SE, *: $p<0.05$, **:$p<0.005$, ***:$p<0.0005$

d) Memory markers

Upon antigen exposure and activation, naive Vγ9Vδ2 T cells ($CD27^+CD45RA^+$) can sequentially differentiate into T_{CM} ($CD27^+CD45RA^-$), T_{EM} ($CD27^-CD45RA^-$), and T_{EMRA} ($CD27^-CD45RA^+$) cells. $CD45RA^-CD27^-$ T_{EM} cells show the highest IFN-γ

secretion, while $CD45RA^{+}CD27^{-}$ T_{EMRA} cells are characterized by a strong cytotoxic activity. In contrast, naive $CD45RA^{+}CD27^{+}$ Vγ9Vδ2 T cells display very low, if any, functional activity. Figure 4.5 describes panel used to identify memory markers.

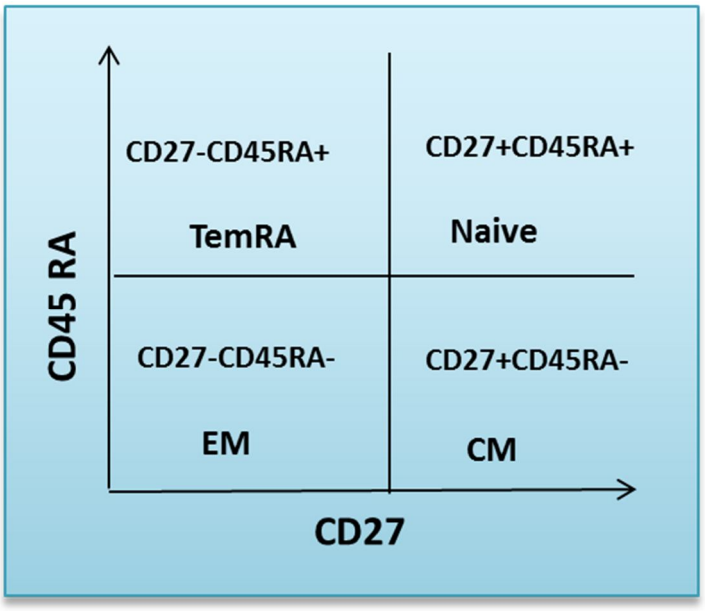


Figure 4.5 Memory marker's classification CD27 and CD45RA markers are used to discriminate between naïve and memory markers on immune cells. The four quadrants indicate different memory markers according to presence or absence of CD27 and CD45 RA

T–ALL patients at diagnosis showed significantly low number of Naïve and Central Memory (CM) cells as compared to patients at remission as well as healthy individuals. Patients at diagnosis express significantly high number of Effector Memory (EM) cells. Patients at remission showed significant high numbers of terminally differentiated (TemRA) cells compared to patients at diagnosis. As the naïve and TemRA cells are significantly less at diagnosis as compared to that of patients at remission and in HI, there is low potential for the immune cells to proliferate and show effector functions in these patients (Figure 4.6).

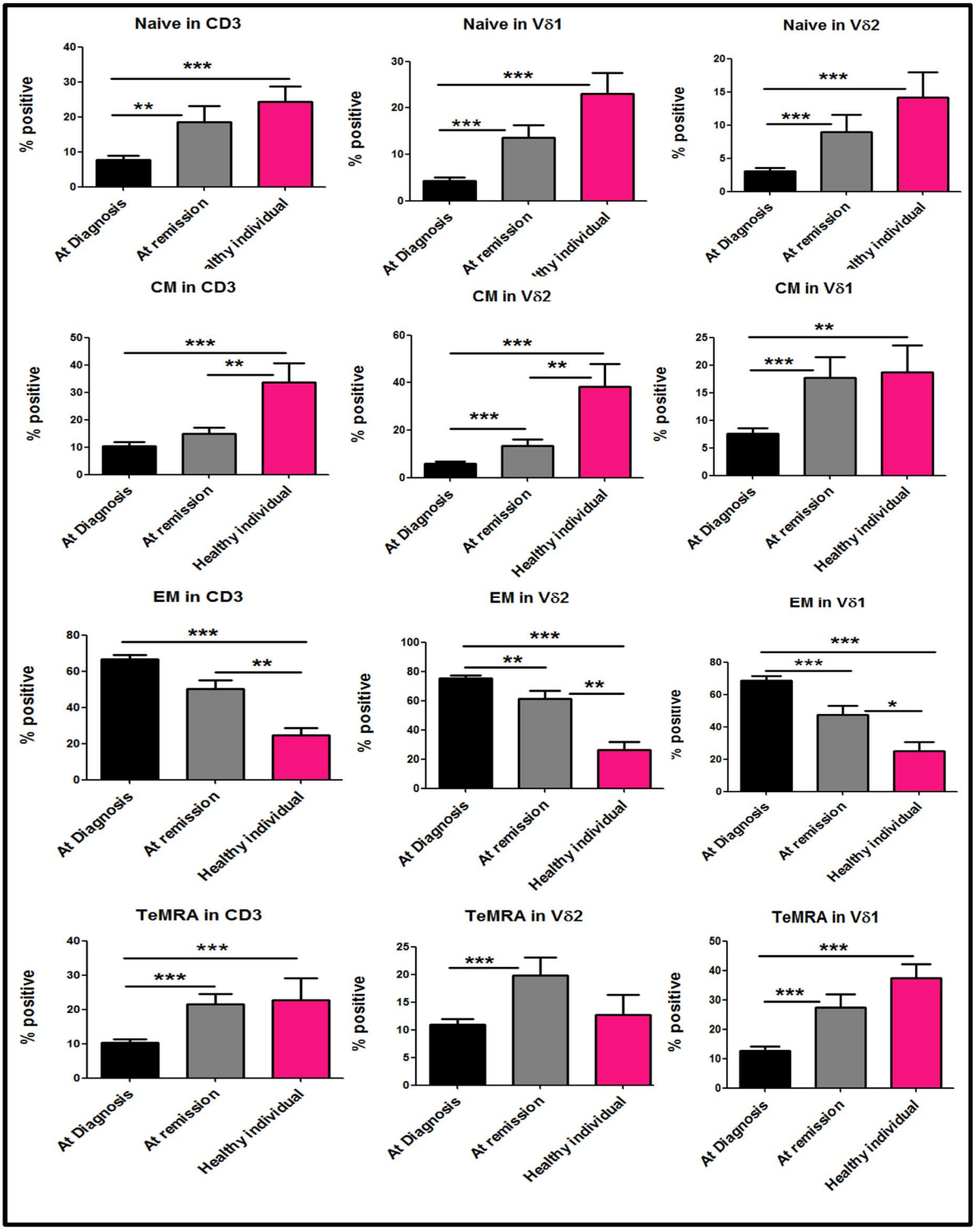


Figure 4.6 Expression of memory markers on CD3, Vδ2 and Vδ1 T cell subsets in T-ALL patients compared to HI. Figure represents % positive expression of memory markers (Naïve, central memory, effector memory, and terminally differentiated) in T-ALL patients at diagnosis (black bar), at remission (gray bar) as compared to healthy individuals (pink bar). Data is represented as mean±SE, *: p<0.05, **:p<0.005, ***:p<0.0005

e) NK cell markers

NKG2D (Natural killer group 2, member D) and CD16b (FCγIIIR) receptor expressions were studied in T-ALL patients at diagnosis, in remission and HI. Patients at diagnosis expressed significantly low number of NKG2D positive cells as compared to HI. Patients at remission showed significantly increased levels of NKG2D and CD16b receptors as compared to patients at diagnosis. Patients in remission showed comparable levels of NKG2D to that of HI. Expression of CD16b on Vδ2 T cells was significantly high in patients in remission compared to HI (Figure 4.7).

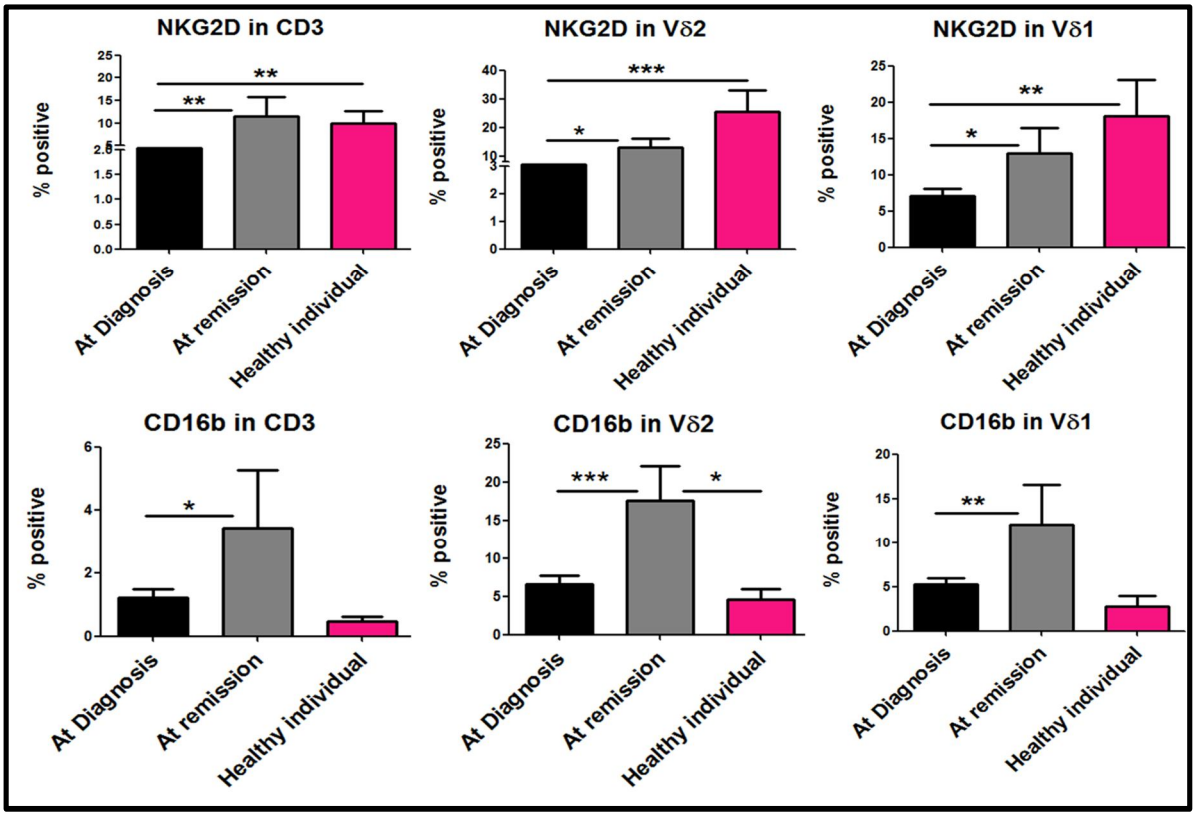


Figure 4.7 Expression of NK markers on CD3, Vδ2 and Vδ1 T cell subsets in T-ALL patients compared to HI. Figure represents % positive expression of NKG2D and CD16b in T-ALL patients at diagnosis (black bar), at remission (grey bar) as compared to healthy individuals (pink bar). Data is represented as mean±SE, *: p<0.05, **:p<0.005, ***:p<0.0005

f) Perforin expression

Perforin is lytic molecule expressed by cytotoxic T cells. We studied the intracellular expression of perforin molecules in T-ALL patients at diagnosis, at remission and HI. T-ALL patients at diagnosis showed significantly reduced numbers of perforin molecules in CD3 T cells compared to HI. Patients in remission showed significant increase in expression of perforin molecules in CD3, Vδ2 and Vδ1 T cell subsets compared to patients at diagnosis. Patients in remission expressed significantly higher perforin molecules in Vδ1 T cells compared to healthy individuals, as shown in figure 4.8.

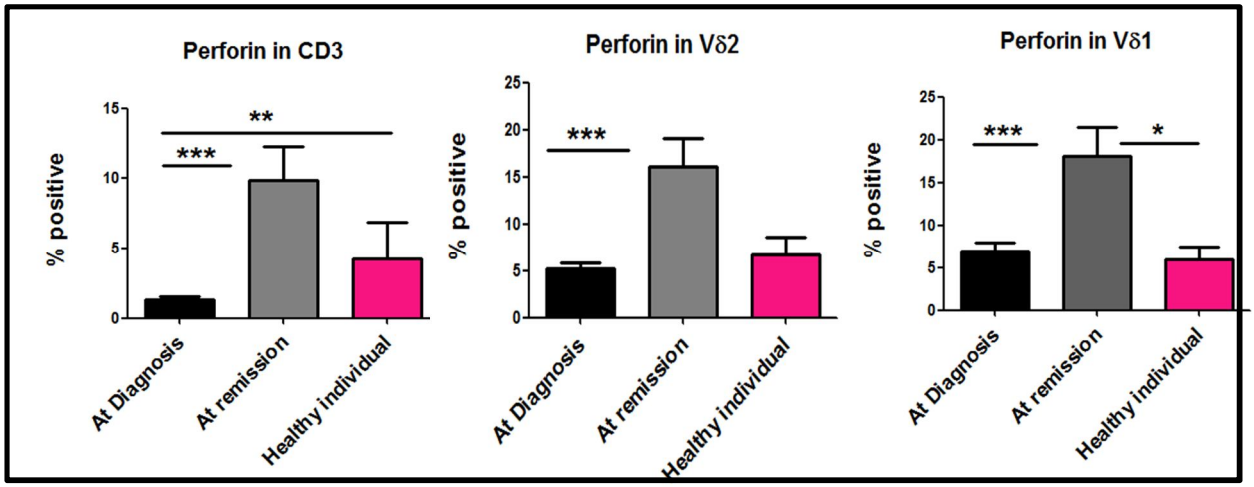


Figure 4.8 Expression of perforin on CD3, Vδ2 and Vδ1 T cell subsets in T-ALL patients compared to HI. Figure represents % positive expression of perforin molecules in T-ALL patients at diagnosis (black bar), at remission (gray bar) as compared to healthy individuals (pink bar). Data is represented as mean±SE, *: $p<0.05$, **:$p<0.005$, ***:$p<0.0005$

Overall it was observed that T–ALL patients at diagnosis showed significantly low numbers of immune cells (CD3, CD4, CD8, NK, Vδ2 T, Vδ1 T, αβ T cells) compared to patients at remission and healthy individuals. Patients in remission showed significantly improved numbers of effector immune cells over patients at diagnosis. Although an improvement was noted in the immune scenario of patients in remission, this was not always comparable to that observed in HI.

4.3 Isolation of γδ T cells

γδ T cells are present in peripheral blood in minor proportion ranging from 1-10%. Hence these cells were enriched before isolating directly from peripheral blood. Briefly, 10-12x10^6 PBMCs were stimulated with 1 nM/ml HDMAPP (analog of IPP) in 24 well plate (Nunc, Denmark). Cells were supplemented with rIL-2 every 3rd day and sub-cultured on day 6. On day 12, cells were harvested and γδ T cells were purified using immunomagnetic purification as described by Dhar et al and Gogoi et al [23, 156]. In order to check the purity of γδ T cells, cells collected from the positive fraction after immunomagnetic purification were analysed for the expression of γδ-TCR by flow cytometry, and it was >95% (Figure 4.9).

Table 4.1 summarizes the total expansion of lymphocytes from healthy individuals (n=25) using HDMAPP and rIL-2. Percentage of purified γδ T cells obtained from the same individuals after expansion is shown in table 4.1. Expansion of PBMCs on day 12 ranged from 7x10^6 to 58.4x10^6 with median of 35x10^6 from a starting population of 10x10^6 PBMCs. The immunomagneticaly purified γδ T cells ranged from 3.75 to 54 x10^6 with a median of 23.1 x10^6. The % yield of purified γδ T cells was in the range of 25.19-94.58% with a median yield of 68.07%. The purity of γδ T cells labelled with FITC fluorochrome was analysed on FACS Aria and it was >95%. These purified γδ T cells were used for cytotoxicity assays, immune synapse studies, co-culture assays and CFSE assays.

Table 4.1: Generation of γδ T cell lines from healthy PBMCs

Healthy Donor	**PBMC count Day '0'**	**Expanded Cell count Day '12'**	**Count of γδ T cells Day '12'**	**% Yield of γδ T cells**
H1	12x10^6	24.25x10^6	19.25x10^6	79.38
H2	12x10^6	16x10^6	6.25x10^6	39.6
H3	12x10^6	35x10^6	31.5x10^6	90
H4	12x10^6	15x10^6	7x10^6	46.6

H5	$12x10^6$	$7x10^6$	$3.75x10^6$	53.57
H6	$12x10^6$	$46x10^6$	$23.1x10^6$	50.21
H7	$12x10^6$	$44x10^6$	$23.4x10^6$	53.18
H8	$12x10^6$	$40x10^6$	$35.75x10^6$	89.37
H9	$12x10^6$	$30x10^6$	$9x10^6$	30
H10	$12x10^6$	$32.75x10^6$	$8.25x10^6$	25.19
H11	$12x10^6$	$50x10^6$	$45x10^6$	90
H12	$12x10^6$	$10.5x10^6$	$7.8x10^6$	74.28
H13	$12x10^6$	$42x10^6$	$24x10^6$	57.14
H14	$12x10^6$	$21.25x10^6$	$18.5x10^6$	87.05
H15	$12x10^6$	$58.4x10^6$	$54x10^6$	92.3
H16	$12x10^6$	$34.5x10^6$	$14.25x10^6$	41.3
H17	$12x10^6$	$30x10^6$	$25x10^6$	83.3
H18	$12x10^6$	$35.25x10^6$	$15.25x10^6$	43.3
H19	$12x10^6$	$33.75x10^6$	$20x10^6$	59.25
H20	$12x10^6$	$32x10^6$	$16.9x10^6$	52.81
H21	$12x10^6$	$40x10^6$	$27.5x10^6$	68.75
H22	$12x10^6$	$42.5x10^6$	$40.2x10^6$	94.58
H23	$12x10^6$	$41.5x10^6$	$28.25x10^6$	68.07
H24	$12x10^6$	$38.5x10^6$	$32.75x10^6$	85.06
H25	$12x10^6$	$37x10^6$	$30.5x10^6$	82.43

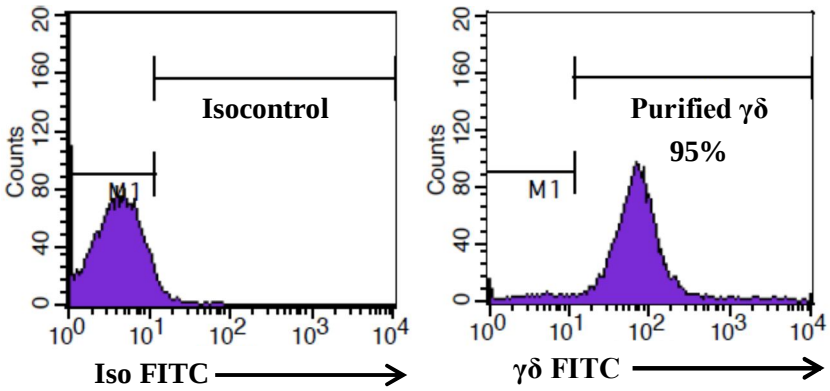


Figure 4.9: Purity of immunomagnetically separated γδ T cells

4.4 Ability of γδ T cells to kill leukemic targets

Immunophenotyping data showed that γδ T cells appear to be an important phenotype that is elevated in T-ALL patients who achieved remission. This underscores the role of these cytotoxic T cells in killing of leukemic blasts. Role of γδ T cells in killing of solid tumors has been reported [23, 172, 208] but their role in killing of leukemic blast cells is not well understood. Hence we studied the ability of γδ T cells to kill leukemic blasts of T-ALL patients.

Cytotoxic potential of purified γδ T lymphocytes isolated from HI, against leukemic blast cells as a target was studied using Chromium Release Assay. Leukemic blasts were treated with Zoledronate for 16-18 hrs or kept untreated, as described in chapter 3. This treatment upregulates IPP in tumor cells and makes them susceptible to lysis by γδ T cells.

It was observed that % cytotoxicity increases with increasing effector: target ratio, with maximum cytotoxicity at 40:1 ratio, as shown in figure 4.10. Patient's leukemic blasts were killed efficiently by effector γδ T lymphocytes. Compared to untreated leukemic blasts, Zoledronate treated leukemic blasts were killed efficiently by γδ T cells and % cytotoxicity was significantly higher at E:T of 40:1 to 10:1 over untreated blasts.

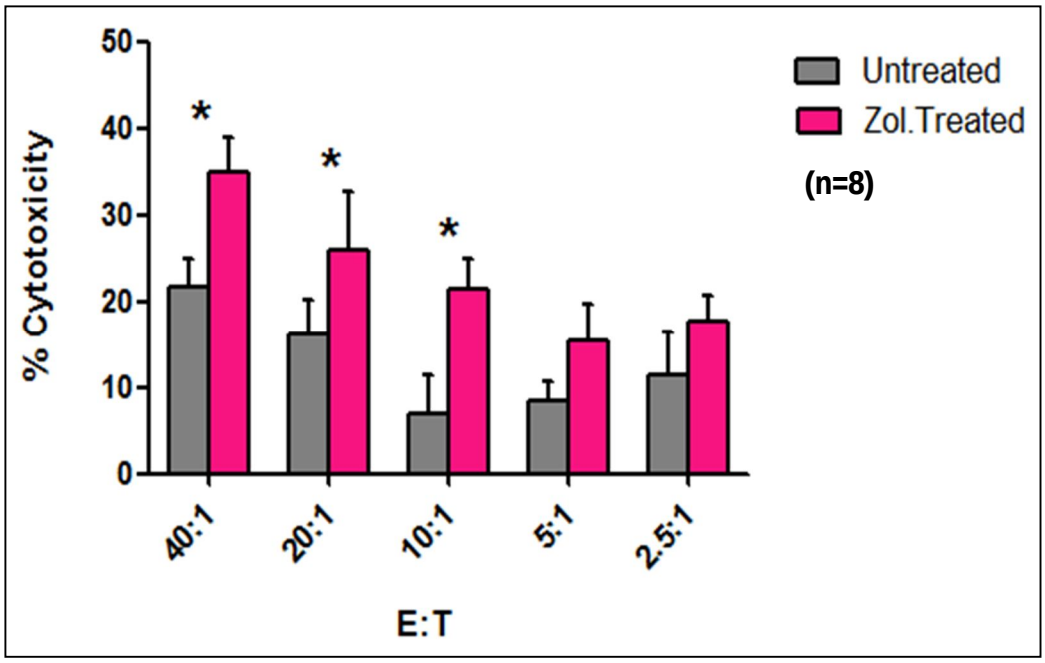


Figure 4.10 Cytotoxic potential of γδ T cells against leukemic blast cells. % cytotoxicity of γδ T cells against leukemic blast cells is measured using four hour ^{51}Cr release assay. Leukemic blasts were treated with zoledroante (pink bars) or kept untreated (grey bars) and were labelled with ^{51}Cr. Leukemic blasts were co-cultured with γδ T cells at different E:T ratios from 40:1 to 2.5:1. X-axis indicates Effector: Target ratio. Data is represented as mean±SE, *:$p<0.05$

4.5 Molecules involved in the killing of leukemic targets

In order to identify the molecules involved in aminobisphosphonate-mediated tumor cell susceptibility to γδ T cell lysis, leukemic blast cells pretreated with Zoledronate were co-cultured with γδ T cells treated with blocking antibodies against γδ TCR, and NKG2D in a 4-hour ^{51}Cr release assay. NKG2D is C-type lectin receptor expressed on γδ T cells and NK cells.

Lysis of Zoledronate-treated leukemic blast cells was inhibited by blocking γδ T cells with anti-γδ TCR mAb (51.67 % inhibition), and anti-NKG2D mAb (19.66 % inhibition). Treatment of γδ T cells with a combination of anti-γδ mAb and anti-NKG2D mAb further reduced the cytotoxicity with 57.75% inhibition (Figure 4.11). γδ T cells incubated with

isotype IgG control antibody did not inhibit lysis of Zoledronate treated Leukemic blasts by γδ T cells, indicating the specificity of the inhibition obtained with the mAbs used in the assay.

Hence, γδ TCR and NKG2D receptor play important role in recognition and killing of leukemic blasts by γδ T cells.

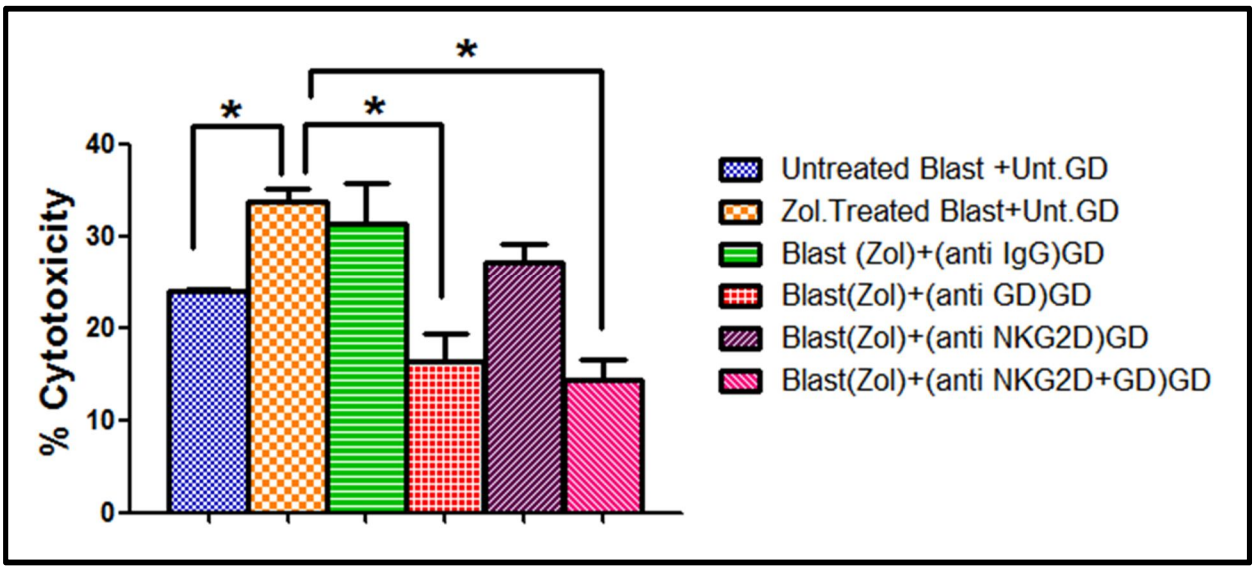


Figure 4.11 Molecules involved in cytotoxic potential of γδ T cells against leukemic blast cells. Zoledronate treated or untreated leukemic blasts were incubated with γδ T cells at an E:T ratio of 30:1 and the percentage of specific lysis determined by ^{51}Cr release assay. γδ T cells were untreated or treated with anti-γδ TCR (10 µg/ml), and anti-NKG2D (20 µg/ml) mAb or a combination of anti-γδ TCR (10 µg/ml) and anti-NKG2D (20 µg/ml) mAbs. As control, γδ T cells treated with isotype specific antibodies were used as effectors and co-cultured with leukemic blasts. Error bars indicate % cytotoxicity±SE. Statistical significance: *, $P < 0.05$. Data is representative of three independent experiments.

4.6 Immune synapse formation between γδ T cells and leukemic targets

Immune synapse is a specialized structure that forms when T lymphocyte and an antigen presenting cell comes in close contact to facilitate communication and signalling. Immune synapse has an ordered structure and these synapses are essential for immune cell activation, function and homeostasis. We observed that γδ T cells recognise Zoledronate treated leukemic blasts and can efficiently kill these blasts (Figure 4.10, 4.11). The interaction of γδ

T cells with leukemic blasts was further assessed by analysing the immune synapse formation.

a) F-Actin polarization at the immune synapse between leukemic blast cells and HI γδ T cells

An immunological synapse is defined as a cell conjugate that demonstrates polarization of F-actin at the cell contact point. Untreated and Zoledronate treated leukemic blasts of T-ALL patients were co-cultured with γδ T cells at 1:2 ratio. As shown in figure 4.12, Zoledronate treated leukemic blasts show significantly increased F-actin polarization at the immune synapse with γδ T cells as compared to F-actin polarization observed with untreated leukemic blasts.

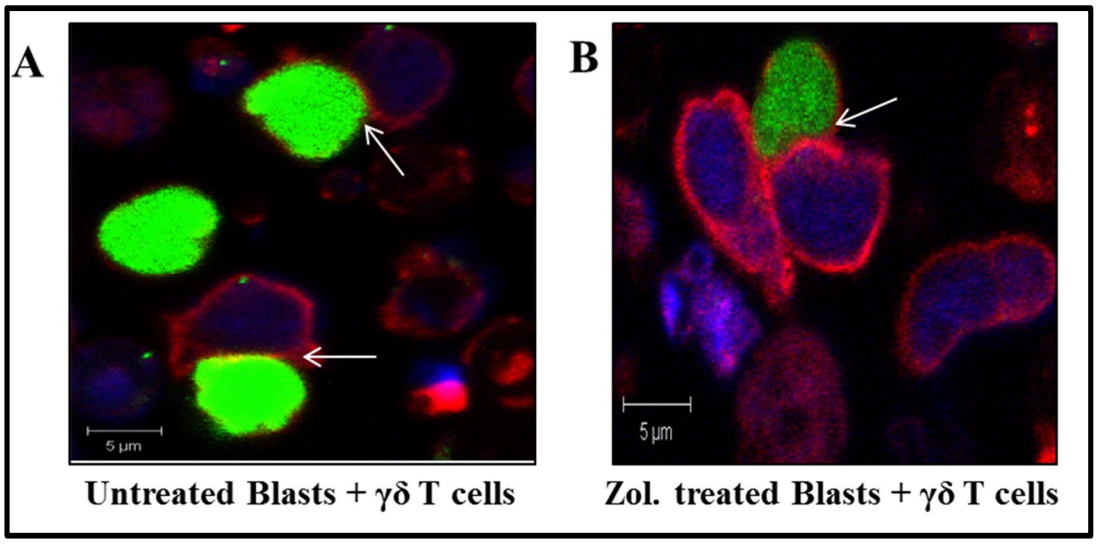


Figure 4.12 Expression of F-actin at immune synapse between γδ T cells and leukemic blasts. Leukemic blasts (Calcein AM, green) were co-cultured with γδ T cells (CMAC blue) at 1:2 ratio. Conjugates were stained for f-actin (phalloidin TRITC, red). A: Untreated blasts co-cultured with γδ T cells, B: Zoledronate treated leukemic blasts co-cultured with γδ T cells. Figure is representative of three independent experiments.

b) F-Actin polarization at the immune synapse between leukemic blast cells and autologous γδ T cells

γδ T cells were isolated from T-ALL patients in remission and were used for immune synapse study. Untreated and Zoledronate treated leukemic blasts of the same T-ALL patients were co-cultured with these autologous γδ T cells at 1:2 ratio. As shown in figure 4.13, Zoledronate treated leukemic blasts show slightly increased F-actin polarization at the immune synapse with γδ T cells as compared to F-actin polarization observed with untreated leukemic blasts. The F-actin polarization observed with Zoledronate treated leukemic blasts and γδ T cells from remission patients was of lesser magnitude compared to that with γδ T cells of HI. The results indicate that γδ T cells of remission patients do not form better immune synapse with leukemic blasts as observed with γδ T cells of HI.

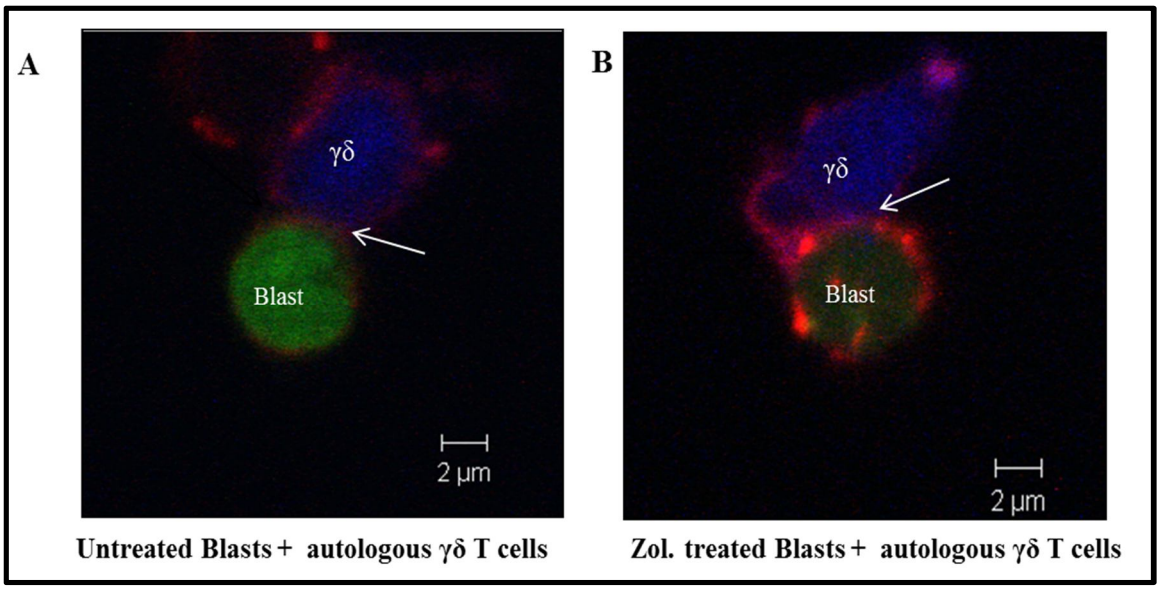


Figure 4.13 Expression of f-actin at immune synapse between autologous γδ T cells and leukemic blasts. Leukemic blasts (Calcein AM, green) were co-cultured with autologous (same patient at remission) γδ T cells (CMAC blue) at 1:2 ratio. Conjugates were stained for f-actin (phalloidin TRITC, red). A: Untreated blasts co-cultured with γδ T cells, B: Zoledronate treated leukemic blasts co-cultured with γδ T cells. Figure is representative of three independent experiments.

c) Release of Perforin at the synapse

Cytotoxic T lymphocytes like γδ T cells secrete perforin and granzyme to kill the tumor targets. We studied the release of perforin molecules by γδ T cells when they are co-cultured with untreated or Zoledronate treated leukemic blasts. γδ T cells when co-cultured with untreated leukemic blasts, showed less release of perforin molecules at immune synapse. Increased expression of perforin at the immune synapse was observed when Zoledronate treated blasts were co-cultured with γδ T lymphocytes (Figure 4.14).

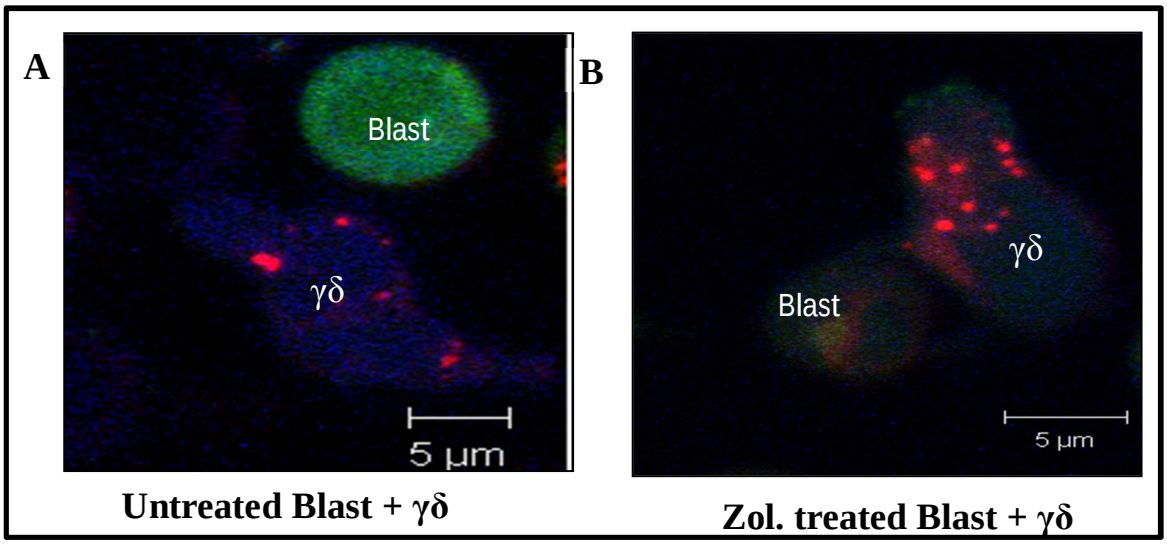


Figure 4.14 Expression of perforin at immune synapse between γδ T cells and leukemic blasts. Leukemic blasts (Calcein AM, green) were co-cultured with γδ T cells (CMAC blue, Perforin, Lysotracker red) at 1:2 ratio, Perforin molecules were stained with specialised dye, Lysotracker red. Release of perforin at immune synapse was studied using live cell imaging on LSM780 confocal microscope. Figure is representative of three independent experiments. A: Untreated blasts co-cultured with γδ T cells, B: Zoledronate treated leukemic blasts co-cultured with γδ T cells.

4.7 Expression of antigens on leukemic blasts of T-ALL patients before and after Zoledronate treatment

γδ T cells recognize and respond to a broad range of antigens, including nonclassical MHC molecules, heat shock proteins, lipids and phosphoantigens. γδ T cells discriminate transformed tumor cells from healthy cells by the upregulation of self-antigens on tumor cells like heat shock proteins (HSP). The expressions of these proteins are increased in tumor cells due to higher metabolism and serves as endogenous danger signals [131]. UL16 binding protein (ULBP) and MHC class I polypeptide-related sequence A and B (MICA, MICB) interact with NKG2D or TCR on Vδ1 γδ T cells and induce their activation. Activated leukocyte cell adhesion molecule (ALCAM/CD166) interact with γδ T cells through CD6 receptor and is involved in γδ T cell activation. Expression of MIC A, MIC B, HSP 60, HSP 70, ULBP-1 and CD166 on untreated and Zoledronate treated leukemic blasts was studied using flow cytometry (Figure 4.15). There was increased expression of MIC A and B, ULBP-1 and CD166 on leukemic blasts after Zoledronate treatment compared to untreated leukemic blasts. Expression of HSP 60 and HSP 70 was unaltered on leukemic blasts after Zoledronate treatment compared to untreated blasts. Expression of CD166 was found to be significantly increased after treatment with Zoledronate as compared to untreated blasts, indicating its role as one of the essential ligand of γδ T cells (Figure 4.15).

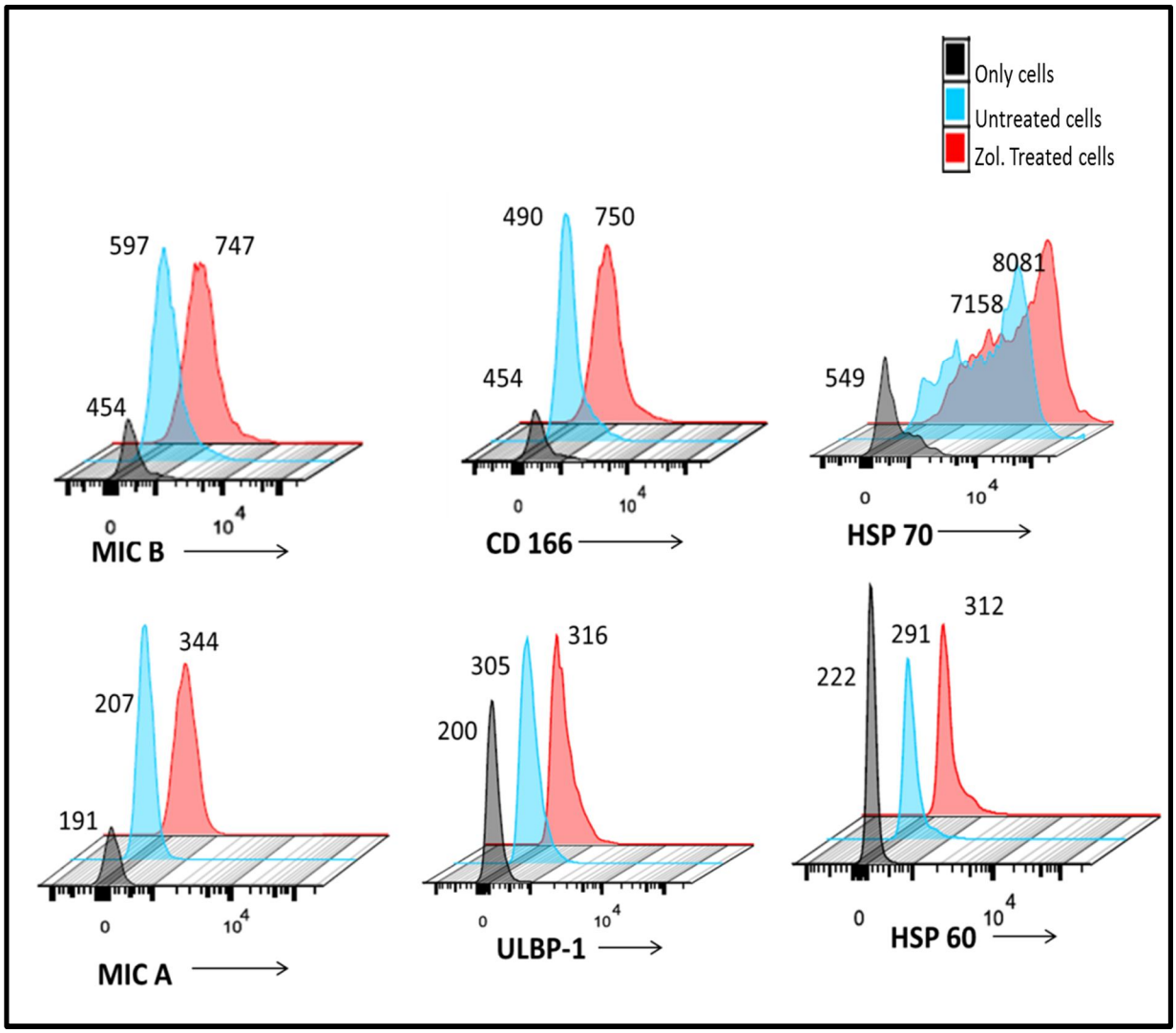


Figure 4.15 Expression of antigens on leukemic blasts of T-ALL patients before and after Zoledronate treatment Median fluorescence intensity (MFI) of different antigens on untreated leukemic blasts (blue), Zoledronate treated leukemic blasts (red) and isocontrol (black). Numbers on top of each histogram indicate MFI of respective cell antigen. Figure is representative of three independent experiments.

4.8 Expansion of Vδ2 T cells after co-culture of leukemic blasts with healthy PBMCs

Ability of leukemic blasts to expand Vδ2 T cells after co-culture with healthy PBMCs was studied using co-culture experiment and CFSE assay.

a) Co-culture assay

Leukemic blasts ($0.1x10^6$) from T-ALL patients were co-cultured with healthy PBMCs in 1:10 ratio in the presence or absence of rIL-2 on day 0. On day 12, cells were harvested and stained with anti Vδ2 PE and CD3 FITC and were acquired on FACS Aria. PBMCs with or

without addition of rIL-2 on day 0 served as baseline control. Incubation of leukemic blasts with PBMCs (1:10) led to outgrowth of CD3Vδ2 T cells (9.62%) that was further expanded in the presence of rIL-2 (24.8%) (Figure 4.16). It indicates that antigens expressed on leukemic blasts are stimulating the outgrowth of Vδ2 T cells and IL-2 is required to further their expansion.

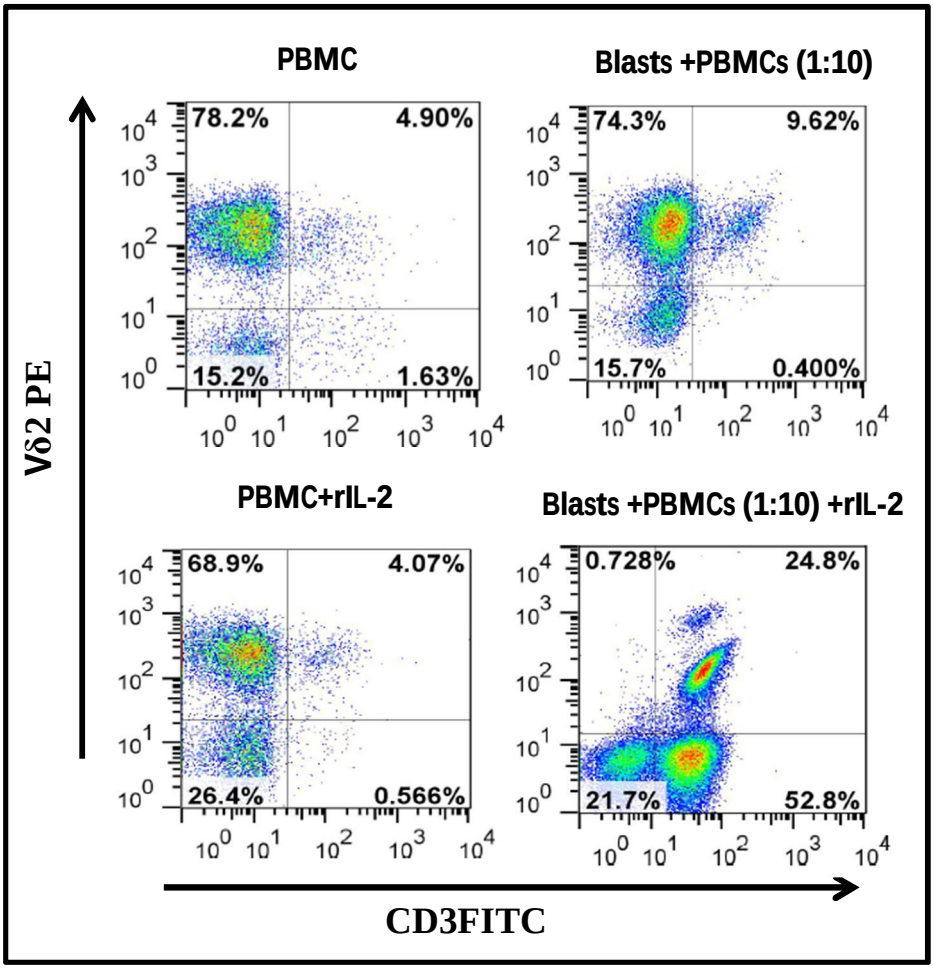


Figure 4.16 Expression of CD3/Vδ2 T cells after co-culture of HI PBMCs with leukemic blasts of T-ALL patients. HI PBMCs were co-cultured with leukemic blasts at 1:10 ratio with or without addition of rIL-2 on day 0. Outgrowth of Vδ2 T cells was studied using flow cytometry. FSC represents CD3 FITC and SSC represents Vδ2 PE. Numbers in each quadrant represent percent positive cells. Figure is representative of three independent experiments.

b) CFSE assay

In CFSE assay, leukemic blasts were co-cultured with healthy PBMCs, which were previously labelled with CFSE (as described in materials and methods) at 1:10 ratio in

presence of BrHPP (200 nM) and rIL-2 (30 IU). PBMCs with rIL-2 served as baseline control and PBMCs with rIL2 and BrHPP served as positive control. BrHPP is a synthetic analog of IPP, a known antigen that drives proliferation of γδ T cells. On day 6, culture was harvested and dual color immunophenotyping was carried out using CFSE/Vδ2 PE and CFSE/αβ PE. As shown in figure 4.17, it was observed that leukemic blasts specifically stimulated the proliferation of Vδ2 T cells (53.9%) which was more as compared to only rIL-2 (12.7%) and rIL-2 and BrHPP together (38.3%). There was a negligible proliferation of αβ T cells when co-cultured with leukemic blasts. Hence the antigens expressed on leukemic blasts specifically stimulate and expand Vδ2 T cells from HI PBMCs.

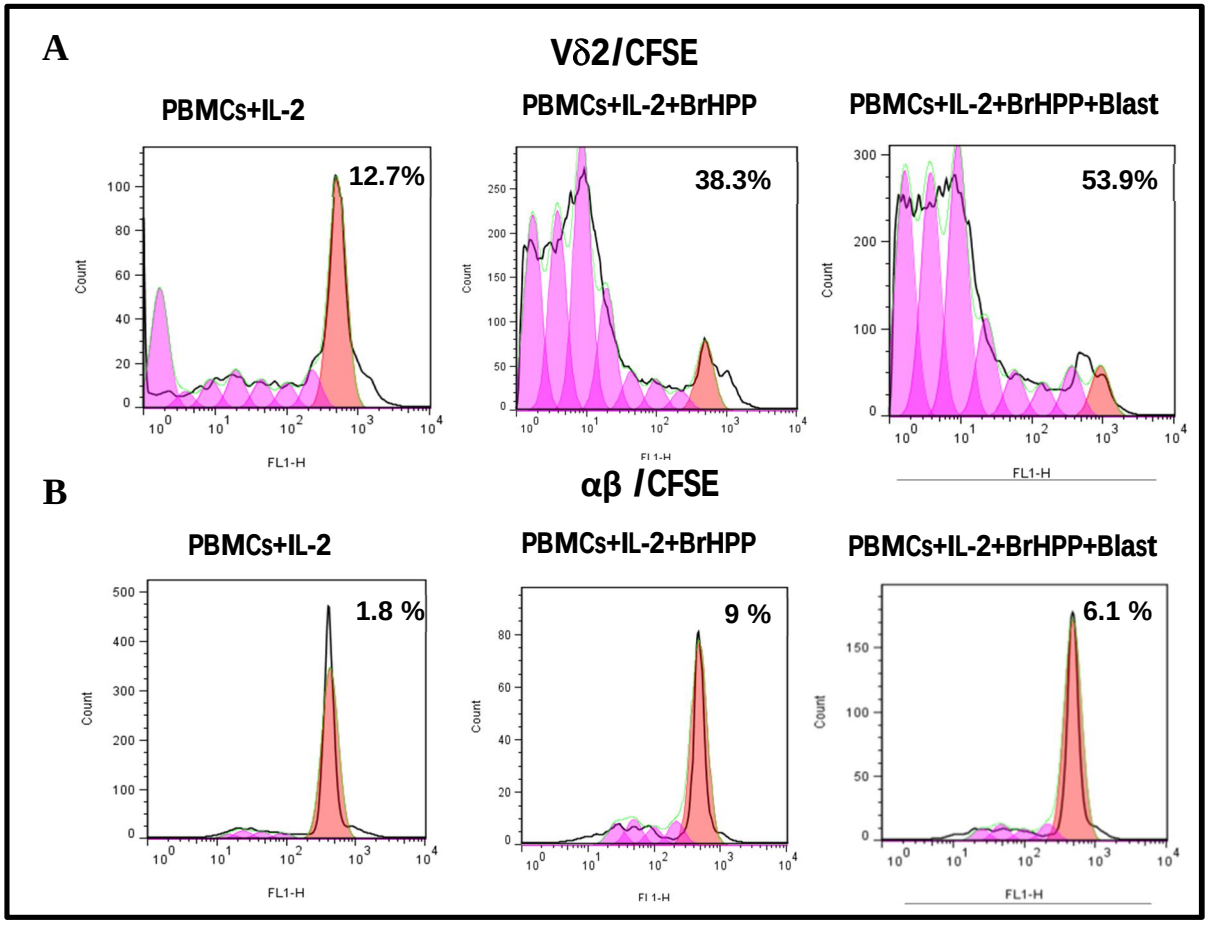


Figure 4.17 Proliferation of Vδ2 T cells in CFSE assay. A. Proliferation of Vδ2 T cells after co-culture of HI PBMCs with leukemic blasts. Numbers in upper right of the panels represent percent divided Vδ2 T cells. B. Proliferation of αβ T cells after co-culture of HI PBMCs with leukemic blasts. Numbers in upper right of the panels represent percent divided αβ T cells. Figure is representative of three independent experiments.

In conclusion, T-ALL patients at diagnosis have significantly low number of immune effector cells as compared to patients at remission and healthy individuals. γδ T cells exhibit robust anti-leukemic activity by killing the leukemic blasts through efficient immune synapse formation.

CHAPTER 5

Result-II

Genomic studies in T-ALL patients exhibiting TCR γδ and TCR αβ gene rearrangement

In our earlier study on Indian T-ALL patients, we identified clonal TCR γ and δ junctional gene rearrangement status of T-ALL as a prognostic marker using a sensitive PCR-coupled Heteroduplex assay [18]. Clinically, survival probability was found to be significantly higher in TCRγδ+ as compared to TCRαβ+T-ALL patients when γ and δ gene rearrangement was considered as prognostic variable [19, 20]. This observation provided an impetus to investigate how TCRγδ+ T-ALL differs biologically from TCRαβ+ T-ALL subgroup.

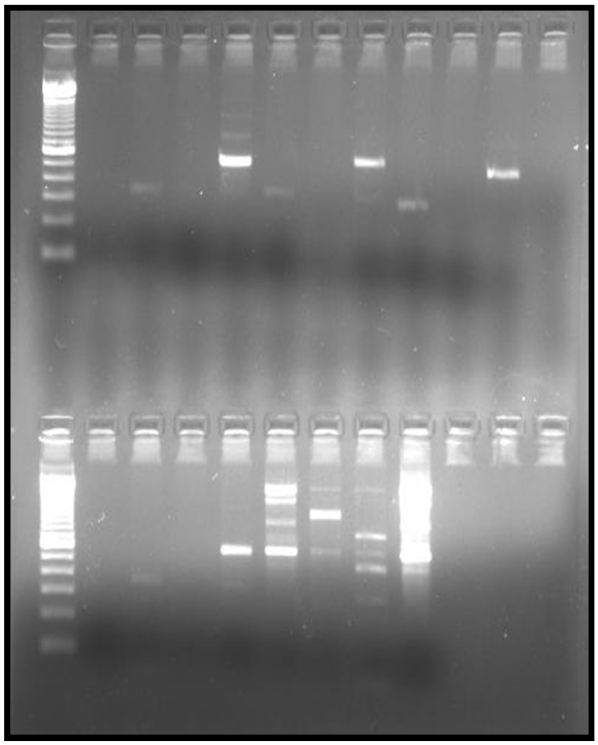
We compared gene expression profile of TCRγδ+T-ALL versus TCRαβ+T-ALL subgroup to identify genes/pathways that provide survival advantage to TCRγδ+T-ALL patients. Further, we also analysed mutation and copy number variations (CNV) in genes (*NOTCH1, IKZF3, BRAF, CDKN2A/B* and *P53*) that were associated with survival/chemoresistance and whose gene expression levels were unaltered in microarray analysis. These markers may provide leads for further risk based stratification of treatment modalities.

5.1 Clonality of leukemic blasts from T-ALL patients

DNA was extracted from PBMCs of T-ALL patients (n=70) using Qiagen DNeasy kit. PCR coupled heteroduplex analysis was performed to study the TCR clonality.

a) Analysis of TCR gamma and delta gene rearrangement

All the possible TCR gene rearrangements in variable, diversity and junctional region were studied in DNA samples from T-ALL patients. Amongst TCR γ gene family, VγI-Jγ1.3/2.3 sequences were most utilized (60%) while from TCR δ repertoire Vδ1-Jδ1 sequences were preferentially rearranged (27.14 %). The details of TCR gene rearrangements observed in the T-ALL patient are given in Table 1. PCR products were analysed on 2% agarose gel electrophoresis (Figure 5.1)



Legends: Upper panel	Legends: Lower panel
Lane 1: 100 bp ladder	Lane1: 100 bp ladder
Lane2: negative control	Lane2: negative control
Lane3: VγI-Jγ1.1	Lane3: VγIV-Jγ1.1
Lane4: VγI-Jγ1.2	Lane4: VγIV-Jγ1.2
Lane5: VγI-Jγ1.3	Lane5: VγIV-Jγ1.3
Lane6: VγII-Jγ1.1	Lane6: Vδ1-Jδ1
Lane7: VγII-Jγ1.2	Lane7: Vδ2-Jδ1
Lane8: VγII-Jγ .3	Lane8: Vδ3-Jδ1
Lane9: VγIII-Jγ1.1	Lane9: Dδ-Jδ1
Lane10: VγIII-Jγ1.2	
Lane11: VγIII-Jγ1.3	

Figure 5.1 γδ PCR to study VDJ gene rearrangements in T-ALL patients. PCR was performed to study TCR γδ gene rearrangement. Representative gel image is shown in the figure. Positive PCR products were observed as distinct bands. First lane in the upper and lower panels of gel indicates 100 bp molecular weight markers. Legends of all lanes in gel are given in the right panel of figure.

Table 5.1: TCR gene rearrangements observed in the T-ALL patient's DNA samples

CASE NO.	VγI-Jγ1.1	VγI-Jγ1.2	VγI-Jγ1.3	VγII-Jγ1.1	VγII-Jγ1.2	VγII-Jγ1.3	VγIII-Jγ1.1	VγIII-Jγ1.2	Vγ-III-Jγ1.3	VγIV-Jγ1.1	VγIV-Jγ1.2	VγIV-Jγ1.3	Vδ1-Jδ1	Vδ2-Jδ1	Vδ3-Jδ1	Dδ1-Jδ1
P1	+	+	++	+	-	++	-	-	-	-	-	++	-	+	-	-
P2	-	-	++	+	+	-	-	-	-	-	-	-	++	+	-	-
P3	±	+	++	+	++	-	+	-	+	-	-	-	+	-	-	+
P4	±	-	++	+	++	++	±	-	±	-	-	-	+	-	-	±
P5	±	+	++	++	+	++	++	-	++	-	-	-	++	-	-	-
P6	±	+	++	-	++	++	++	-	-	-	-	±	-	-	-	±
P7	-	-	++	+	+	+	+	-	±	-	-	-	+	-	++	-
P8	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-
P9	+	-	++	+	+	++	-	-	+	-	-	±	-	±	-	±
P10	+	+	++	+	+	++	-	-	-	-	-	-	+	-	-	-
P11	+	-	++	±	+	++	-	-	±	-	-	±	-	±	+	-
P12	±	±	++	+	+	-	-	-	±	-	-	+	+	+	-	-
P13	+	-	++	-	+	++	-	-	-	-	-	-	±	-	-	-
P14	+	+	++	-	-	++	±	-	++	-	-	-	-	±	-	-
P15	+	+	++	-	±	-	-	-	++	-	-	-	+	-	-	-
P16	++	+	+	+	+	-	++	-	++	-	-	-	+	-	-	-
P17	++	+	+	±	±	++	+	-	-	+	-	-	±	±	±	+
P18	+	+	++	±	+	-	±	-	+	+	-	-	++	+	-	-
P19	++	±	++	+	+	+	+	-	+	+	-	+	+	+	-	-

P20	+	-	++	++	-	±	+	-	+	+	-	+	+	+	-	+
P21	++	-	++	±	-	+	+	-	+	±	-	+	-	+	-	+
P22	-	++	++	+	-	-	++	-	++	-	-	-	-	+	-	+
P23	++	-	++	±	-	++	±	-	+	+	-	++	-	-	-	-
P24	-	+	++	±	±	±	-	-	-	±	-	±	+	+	+	±
P25	++	-	++	++	++	++	+	-	+	±	-	±	++	++	-	++
P26	++	-	++	±	±	++	++	-	+	+	-	±	+	+	-	+
P27	+	-	++	-	±	±	±	-	++	-	-	++	+	-	±	-
P28	++	-	++	+	+	+	+	-	±	-	-	-	++	+	+	-
P29	+	-	+	+	+	+	+	-	+	±	-	+	+	+	-	+
P30	+	-	+	-	+	+	++	-	++	-	-	±	+	±	±	±
P31	±	-	+	-	+	+	-	-	+	-	-	++	-	-	+	-
P32	±	-	++	-		±	-	-	-	-	-	-	-	-	±	-
P33	-	-	+	-	+	++	-	-	-	-	-	-	±	-	±	-
P34	±	+	++	±	-	±	+	-	+	-	-	-	-	±	-	-
P35	±	-	++	±	-	-	±	-	+	-	-	-	+	-	-	-
P36	+	-	++	+	-	+	+	-	+	-	-	±	-	-	-	-
P37	+	-	++	-	-	-	±	-	+	-	-	±	+	-	-	+
P38	+	-	++	-	±	-	-	-	-	+	-	-	±	-	-	-
P39	-	-		-	-	±	-	-	-	-	-	±	-	-	-	-
P40	-	-	+	-	-	+	-	-	+	-	-	±	±	-	-	-
P41	+	-	+	-	-	+	±	-	±	-	-	±	-	-	-	-
P42	±	-	+	-	-	±	-	-	+	-	-	±	-	-	-	-
P43	-	-		-	-	-	-	-	-	-	-	-	-	+	-	±
P44	-	-	+	-	-	±	-	-	±	-	-	±	±	-	-	-
P45	+	-	+	±	-	-	+	-	+	+	-	+	-	+	-	+
P46	±	-	±	-	-	±	-	-	-	-	-	±	-	-	-	+
P47	-	-	+	-	-	±	-	-	++	-	-	-	-	-	-	-
P48	-	-	++	-	-	++	-	-	-	-	-	++	±	-	±	+
P49	-	-		-	-	-	-	-	-	-	-	-	-	-	+	-
P50	±	-	±	-	-	-	-	-	±	-	-	-	-	-	-	+
P51	±	-	+	-	-	+	-	-	+	-	-	+	+	-	-	+
P52	-	-		-	-	-	-	-	-	-	-	-	+	-	+	+
P53	+	-	+	-	-	-	+	-	+	-	-	-	+	+	+	+
P54	-	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+
P55	+	-	+	-	-	+	-	-	+	-	-	+	+	+	+	+
P56	±	-	+	+	-	±	+	-	±	+	-	+	+	+	+	+
P57	-	-	+	±	-	+	±	-	+	-	-	+	+	+	+	+
P58	-	-		±	-	-	±	-	-	±	-	-	-	±	-	++
P59	±	-	+	±	-	+	±	-	+	±	-	+	+	+	+	+
P60	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
P61	+	-	+	-	++	++	+	-	++	±	-	+	++	+	-	±
P62	+	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-
P63	+	-	-	++	-	++	+	-	+	-	-	+	++	+	+	+
P64	-	-	-	-	-	-	+	-	++	-	-	-	+	-	+	-
P65	+	-	++	±	-	+	+	-	+	-	-	++	+	±	+	+
P66	-	-	++	±	-	++	±	-	++	-	-	++	+	+	+	+
P67	+	-	++	+	-	+	+	-	+	±	-	+	-	+	-	++
P68	±	-	+	+	-	-	±	-	+	+	-	+	-	+	-	-
P69	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
P70	±	±	++	±	-	+	+	-	+	+	-	+	-	+	±	+

Legends: +: positive, - : negative, ++: strong positive, ±: faint positive

b) Heteroduplex analysis

The positive products obtained from the TCR gamma delta PCR were subjected to heteroduplex analysis and were further analysed on 6% PAGE. The gel was visualised by silver staining. The patient showing positive rearrangement for both TCR gamma and delta region was designated as TCR γδ clonal patient. If the patient showed neither gamma nor delta or either of the two rearrangements, then they were designated as TCR αβ clonal T-ALL patients. Details of rearrangements are given in Table no.5.2. Heteroduplex patterns on silver stained gel are shown in Figure 5.2.

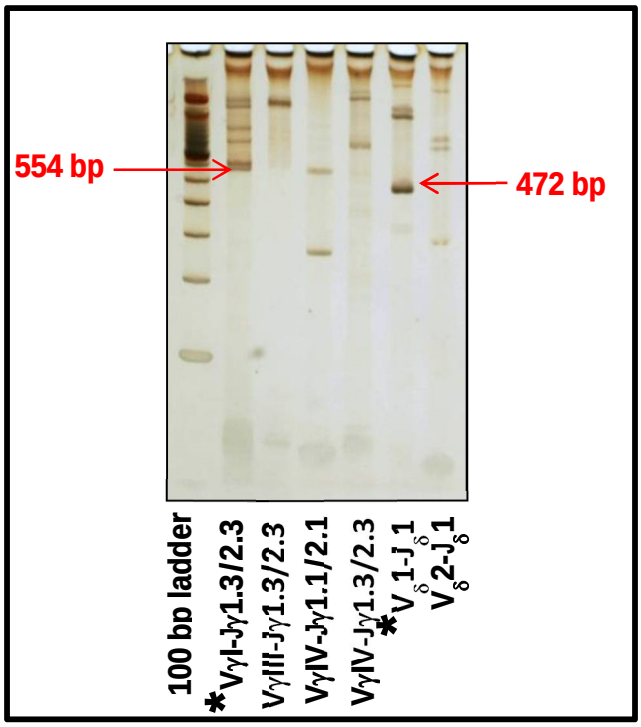


Figure 5.2 Heteroduplex analysis of PCR-positive samples on 6% PAGE visualised using silver staining

Figure shows representative image of TCR γδ clonal T-ALL patient sample. First lane of gel indicates 100 bp molecular weight marker. * indicates dominant TCR gene rearrangements observed in T-ALL patients.

Table 5.2: Heteroduplex analysis performed to confirm the TCR clonality in T- ALL patients

CASE NO.	VγI-Jγ1.1	VγI-Jγ1.2	VγI-Jγ1.3	VγII-Jγ1.1	VγII-Jγ1.2	VγII-Jγ1.3	VγIII-Jγ1.1	VγIII-Jγ1.2	VγIII-Jγ1.3	VγIV-Jγ1.1	VγIV-Jγ1.2	VγIV-Jγ1.3	Vδ1-Jδ1	Vδ2-Jδ1	Vδ3-Jδ1	Dδ-Jδ1
P1	-	-	-	-		+						+		-		
P2			++	-	+	++							++	-		
P3	-	-	++	+	+		-		-				-			+
P4	-		++	+	+	-	-		+				-			+
P5	+	-	++	+	±	++	-		+				+			-
P6	-	-	++		+	+	-					-				-

P7			**++**	**+**	**-**	**+**	**±**		**-**				**-**	**++**	**-**	
P8	-		-												++	
P9	-		++	-	+	+			-			-		-		-
P10	-	-	-	+	+	-							-			
P11	**-**		**+**	**-**	**-**	**+**			**-**			**-**		**-**	**+**	
P12	**-**	**-**	**++**	**+**	**+**				**-**			**-**	**+**	**-**		
P13	**-**		**++**		**-**	**+**							**+**			
P14	-	-	++			++	-		-					-		
P15	**-**	**-**	**+**		**-**				**+**				**±**			
P16	**++**	**-**	**-**	**-**	**-**		**-**		**-**				**+**			
P17	**++**	**-**	**-**	**-**	**-**	**++**	**-**			**-**			**-**	**-**	**-**	**+**
P18	-	-	-	-	-		-		-	-			+	-		
P19	-	-	++	-	-	+	-		-	-		-	-	-		
P20	-		+	++		-	-		-	-		-	-	-		-
P21	**++**		**++**	**-**		**-**	**-**		**-**	**-**		**-**		**-**		**+**
P22		**++**	**++**	**-**			**-**		**-**					**-**		**+**
P23	-		++	-		±	-		±	-		-				
P24		-	++	-	-	-				-		-	-	-	-	-
P25	**+**		**++**	**++**	**-**	**-**	**-**		**-**	**-**		**-**	**-**	**-**		**±**
P26	**-**		**++**	**±**	**-**	**±**	**-**		**-**	**-**		**-**	**-**	**-**		**±**
P27	**+**		**+**		**-**	**-**	**±**		**++**			**++**	**+**		**-**	
P28	**-**		**-**	**+**	**-**	**±**	**-**		**-**				**+**	**-**	**-**	
P29																++
P30					+		+									
P31				**±**								**++**				
P32			++													
P33			**++**			**+**							**+**			
P34		+	++	+		±			±							
P35			**++**	**±**									**+**			
P36						±	±									
P37			**++**						**±**							**+**
P38			++													
P39						±										
P40			**+**										**+**			
P41																
P42			+													
P43														+		
P44			**+**										**+**			
P45							**±**			**+**			**±**			
P46									+			±				
P47																
P48			+			+						+				
P49																
P50			**±**						**±**							**+**
P51			**±**			**±**						**+**	**+**			
P52																
P53			**+**						**+**				**+**			
P54			**+**								**+**		**+**			
P55			+			+						+				
P56							+					+				
P57							**+**		**+**				**+**	**+**	**+**	
P58														+		
P59							-	-	-	-			-	-	-	-
P60	-		+	±		-	-		-	-		-	-	-	-	-
P61	-		-		+	-	±		-			-	-	-		-
P62	-		-	±	-	-	-									

P63	-		++	±		-	-		-			-	-	-	-	-
P64							-		+				-			
P65	-		++	-		-	-		-			-	-	-	-	-
P66			+	±		-	-		±			-	-	-	-	-
P67	**-**		**++**	**-**		**-**	**-**		**±**	**-**		**-**		**±**		**+**
P68	-		-	+			-		-	++		-		-		
P69													+			
P70	-	-	+	-		-	-		-	-		-		-	-	-

Legends: +: positive, - : negative, ++: strong positive, ±: faint positive, Patient IDs marked in red are TCRγδ clonal

Out of 70 T-ALL patients, 29 patients were TCR γδ clonal (41.4%), while 41 patients were found to be TCR αβ clonal (58.6%). Amongst TCR γ gene family, VγI-Jγ1.3/2.3 sequences were most utilized (60%) while from TCR δ repertoire Vδ1-Jδ1 sequences were preferentially rearranged (27.14 %).

5.2 Differential regulation of genes within two T-ALL subtypes

Unsupervised hierarchical tree distinguished two subsets of T-ALL patients i.e. TCRγδ+T-ALL and TCRαβ+T-ALL, showing differential regulation of genes based on 2 fold change in gene expression with $p<0.05$ (Figure 5.3). We identified list of 394 genes with 2 fold change and $p<0.05$. Further, we could identify 3 clusters in these 394 genes, which are strongly correlated with each other within subgroups of T-ALL. These genes were found to be involved in the pathways related to cluster of differentiation, haematopoietic cell lineages, toll-like receptors, cytokine signalling molecules, inflammatory responses and mainly apoptotic pathway, as observed by functional annotation of genes using Gene Ontology, DAVID Bioinformatics Resources and KEGG database (Table 5.3).

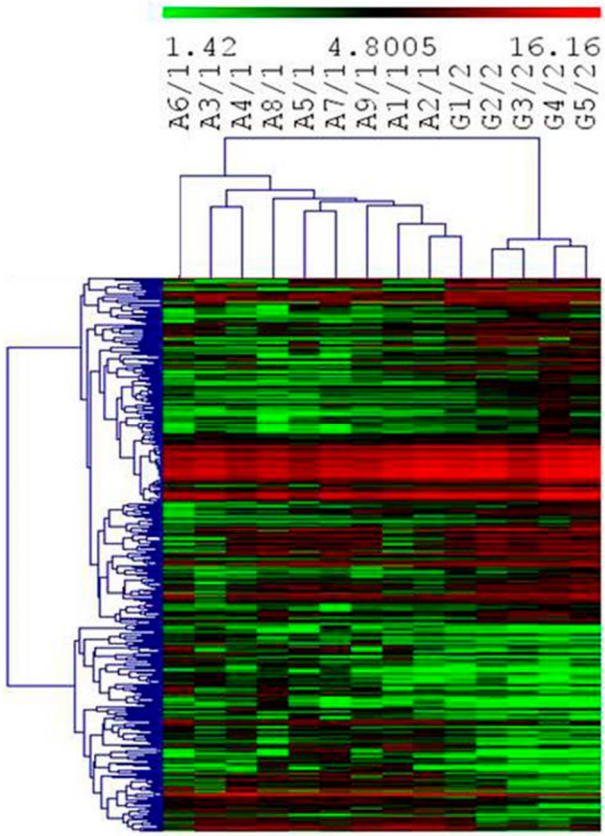


Figure 5.3 Hierarchical clustering of T-ALL patients based on fold changes in gene expression

Heatmap represents unsupervised clustering of 394 genes having 2 fold change in expression and p value <0.05 in T-ALL patients (n=14). Columns represent two subsets of T-ALL patients (A1-A9, TCRαβ+T-ALL, n=9; G1-G5, TCRγδ+T-ALL, n=5) and each row represents a gene. Relative levels of gene expression are depicted with a colour scale, where red represents the high level of expression and green represents the low level.

Table 5.3: Functional classification of the differentially regulated genes in TCRγδ+ T-ALL patients

Term	%	p-value	Genes	Fold Enrichment	Bonferroni	Benjamini	FDR
Upregulated							
Graft-versus-host disease	3.346457	4.05E-14	HS.534322, HS.512576, HS.2200, HS.631991, HS.1051, HS.347270, HS.351279, HS.2007, HS.562457, HS.659860, HS.181244, HS.387679, HS.171182, HS.409934, HS.645228	12.52282	4.98E-12	4.98E-12	4.67E-11

Antigen processing and presentation	3.346457	1.47E-08	HS.534322, HS.512576, HS.631991, HS.85258, HS.14623, HS.347270, HS.436568, HS.351279, HS.562457, HS.512572, HS.659860, HS.181244, HS.387679, HS.409934, HS.645228	5.884215	1.80E-06	9.02E-07	1.69 E-05
Allograft rejection	2.362205	1.77E-08	HS.534322, HS.1051, HS.347270, HS.631991, HS.351279, HS.2200, HS.2007, HS.181244, HS.387679, HS.171182, HS.409934	9.576271	2.18E-06	7.26E-07	2.04 E-05
Type I diabetes mellitus	2.362205	1.05E-07	HS.534322, HS.1051, HS.347270, HS.631991, HS.351279, HS.2200, HS.2007, HS.181244, HS.387679, HS.171182, HS.409934	8.208232	1.29E-05	3.23E-06	1.21 E-04
Cytokine-cytokine receptor interaction	5.511811	2.18E-07	HS.370036, HS.532082, HS.655801, HS.2233, HS.81564, HS.479754, HS.2164, HS.583348, HS.169191, HS.25333, HS.34526, HS.225946, HS.287369, HS.514821, HS.210546, HS.143961, HS.415768, HS.72933, HS.496646, HS.632586, HS.89714, HS.632592, HS.632713, HS.2007, HS.632790, HS.83077, HS.221375, HS.204044	3.070255	2.68E-05	5.35E-06	2.51 E-04
Autoimmune thyroid disease	2.362205	8.92E-07	HS.534322, HS.1051, HS.347270, HS.631991, HS.351279, HS.2200, HS.2007, HS.181244, HS.387679, HS.171182, HS.409934	6.759721	1.10E-04	1.83E-05	0.00 1028

Cell adhesion molecules (CAMs)	3.346457	1.01E-05	HS.370510, HS.534322, HS.150718, HS.631991, HS.521989, HS.85258, HS.347270, HS.173840, HS.351279, HS.49774, HS.133397, HS.181244, HS.387679, HS.171182, HS.643447, HS.409934	3.699923	0.001243	1.78E-04	0.011664
Viral myocarditis	2.362205	2.62E-05	HS.534322, HS.347270, HS.631991, HS.495912, HS.351279, HS.2200, HS.181244, HS.387679, HS.643447, HS.171182, HS.409934	4.855574	0.00322	4.03E-04	0.030234
Natural killer cell mediated cytotoxicity	3.149606	4.60E-05	HS.509513, HS.512576, HS.2200, HS.350581, HS.655801, HS.97084, HS.243564, HS.1051, HS.2007, HS.562457, HS.512572, HS.659860, HS.181244, HS.643447, HS.645228	3.456098	0.005646	6.29E-04	0.053066
Chemokine signaling pathway	3.543307	2.20E-04	HS.134587, HS.514821, HS.395482, HS.370036, HS.143961, HS.72933, HS.81564, HS.632586, HS.515544, HS.2164, HS.481545, HS.89714, HS.632592, HS.169191, HS.34526, HS.391860, HS.83381, HS.225946	2.76534	0.026698	0.002702	0.253357
Intestinal immune network for IgA production	1.771654	2.35E-04	HS.534322, HS.347270, HS.631991, HS.351279, HS.387679, HS.171182, HS.409934, HS.225946	5.276721	0.028498	0.002625	0.270671

Asthma	1.377953	3.86E-04	HS.534322, HS.347270, HS.631991, HS.351279, HS.387679, HS.409934	6.934541	0.046387	0.00395	0.444278
Hematopoietic cell lineage	1.968504	0.002692	HS.534322, HS.25333, HS.133397, HS.218040, HS.632790, HS.85258, HS.2233, HS.249217, HS.479754	3.34056	0.282196	0.025182	3.060389
Systemic lupus erythematosus	1.771654	0.02063	HS.534322, HS.347270, HS.533295, HS.631991, HS.351279, HS.143080, HS.46423, HS.387679, HS.171182, HS.409934	2.61171	0.923002	0.167349	21.36547
Downregulated							
Cytokine-cytokine receptor interaction	2.571429	0.059036	HS.181097, HS.567559, HS.194236, HS.93177, HS.174273, HS.501497, HS.113222, HS.310511, HS.184926	2.104525	0.996914	0.996914	48.77112
Melanogenesis	1.428571	0.074845	HS.591863, HS.247744, HS.472101, HS.1897, HS.269782	3.094195	0.999383	0.975158	57.47667

The data supports deregulation of genes in TCRαβ+T-ALL patients which are mainly involved in leukemogenesis. The gene expression data is deposited in NCBI's Gene Expression Omnibus (GEO, available at: http://www.ncbi.nlm.gov/geo/) and is accessible through GEO series accession number *GSE37389.*

5.3 Differential gene expression related to leukemia

Genes with statistical significant differential expression ($p<0.05$) between TCRγδ+ T-ALL and TCRαβ+T-ALL patients and categorised as "Leukemia associated genes" based on

functions and pathways, using biological interpretation tool Biointerpreter (Genotypic Technology) and GeneSpring Software, were used to generate Heatmap (Figure 5.4).

CD28 and MLLT10 (Myeloid/lymphoid or mixed lineage leukemia) were significantly underexpressed leukemia related genes in TCRγδ+T-ALL patients. *IL6R, IL33, CD80, TLR7, ALOX12, LILRB1, TLX1, IL18, MLLT4, LTC45, MLL3, BAALC, IL10RA, IL28RA, LAIR2, CYSLTR1* were found to be significantly overexpressed in TCRγδ+ T-ALL patients.

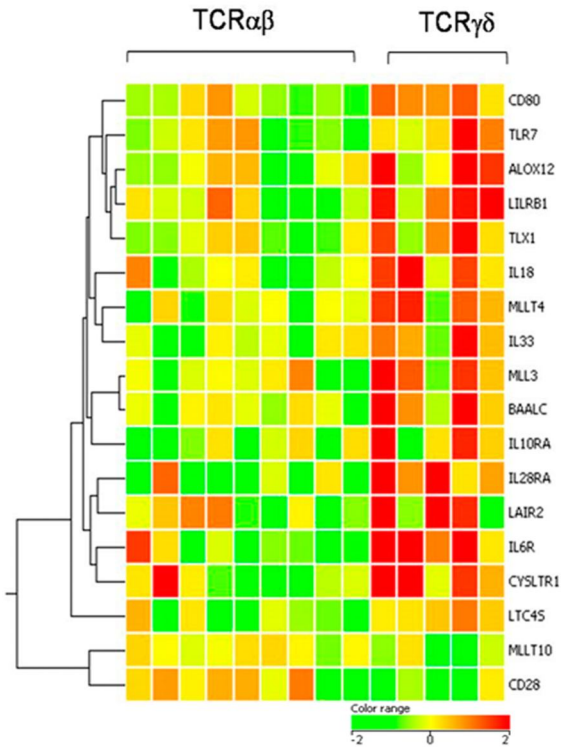


Figure 5.4 Heatmap of leukemia associated genes in T-ALL patients

Heatmap exhibits differential regulation of leukemia associated genes in TCRαβ+T-ALL (n=9) V/s TCRγδ+T-ALL (n=5) patients. These genes were selected on the basis of unsupervised clustering with non-average complete linkage rule and were statistically significant by unpaired students-t test ($p<0.05$). Relative levels of gene expression are depicted with a colour scale, where red represents the high level of expression and green represents the low level.

5.4 Gene expression profile related to apoptosis

Genes with statistical significant differential expression ($P<0.05$) between TCRγδ+ T-ALL and TCRαβ+T-ALL patients and categorised as "Apoptosis related genes" based on functions and pathways, using biological interpretation tool Biointerpreter (Genotypic Technology) and GeneSpring Software, were analysed further. *TNFRSF10C, FASLG, NFKB1* genes demonstrated significant fold change (1.34, 1.08 and -1.59 $\log_2$ respectively) between two

subgroups. Heatmap was generated for a set of 23 apoptosis related genes that were significantly differentially regulated (Figure 5.5).

Out of these set of genes, 21 genes were up regulated in TCRγδ+T-ALL patients, while only 2 genes (SIAH1 and CD28) were down regulated. Increased expression of important genes such as *TNFRSF10B, TNFRSF10C, PHLDA1, NGFR, BBC3, IL18, SOCS2, HRK, BAX* was observed in TCRγδ+T-ALL patients.

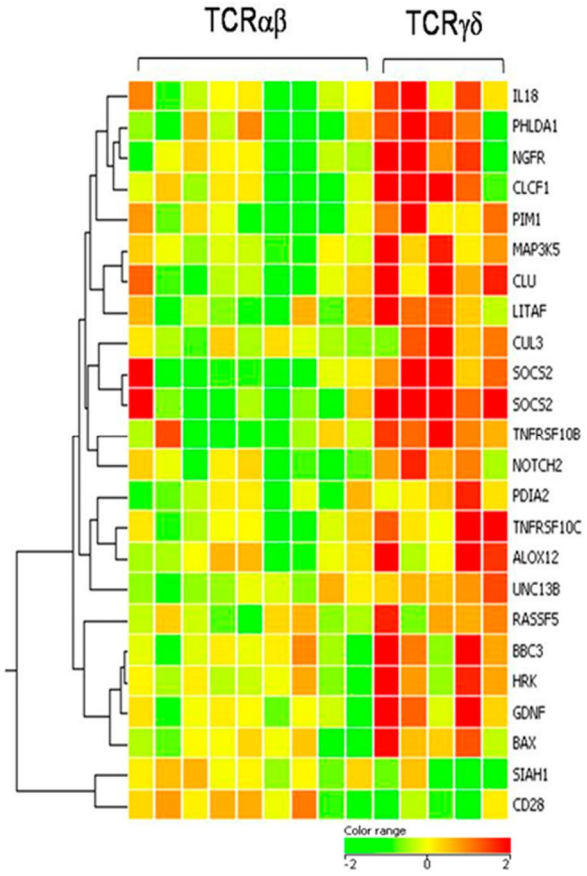


Figure 5.5 Heatmap of apoptosis related genes in T-ALL patients

Heatmap exhibits differential regulation of apoptosis related genes in TCRαβ+T-ALL (n=9) V/s TCRγδ+T-ALL (n=5) patients. These genes were selected on the basis of unsupervised clustering with non-average complete linkage rule and were statistically significant by unpaired students-t test ($p<0.05$). Relative levels of gene expression are depicted with a colour scale, where red represents the high level of expression and green represents the low level.

5.5 Biological association of differentially regulated genes

Consistent with KEGG pathway analysis, based on 2 fold changes and $p<0.05$, upregulated genes in TCRγδ+T-ALL patients belong to biological processes such as antigen processing and presentation (*IFI30, KLRD1, CD74, KIR2DS2*) and cell adhesion molecules (*HLA-DQB1, ICAM1, ITGA6, CD274*; Table 4). *TUBB6* and *PLCB4* of Gap Junction; *CACNA1H,*

HSPA1A, *NFKB1* of MAPK signalling pathway; *IFNB1* and *CISH* of Jak-STAT signalling pathway were found to be down regulated in TCRγδ+T-ALL patients (Table 5.4).

Table 5.4: Biological Annotation of genes with high expression in TCRγδ+ T-ALL /TCRαβ+ T-ALL patients

High expression in TCRγδ+ T-ALL patients			
Gene Symbol	**Gene Name**	**Unigene ID**	**Pathway Name**
HLA-DQB1[a]	Major histocompatibility complex, class II, DQ beta 1	Hs.697047	Type I diabetes mellitus Antigen processing and presentation Cell adhesion molecules (CAMs)
IFI30	Interferon, gamma-inducible protein 30	Hs.14623	Antigen processing and presentation
CD86	CD86 molecule	Hs.171182	Cell adhesion molecules (CAMs) Type I diabetes mellitus
KLRD1	Killer cell lectin-like receptor subfamily D, member 1	Hs.562457	Antigen processing and presentation
PRF1	Perforin 1 (pore forming protein)	Hs.2200	Type I diabetes mellitus
HLA-DQA1	Major histocompatibility complex, class II, DQ alpha 1	Hs.387679	Type I diabetes mellitus Antigen processing and presentation Cell adhesion molecules (CAMs)
ICAM1	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	Hs.707983	Cell adhesion molecules (CAMs)
CD74	CD74 molecule, major histocompatibility complex, class II invariant chain	Hs.436568	Antigen processing and presentation
CD8A	CD8a molecule	Hs.85258	Antigen processing and presentation Cell adhesion molecules (CAMs)
KLRC1	Killer cell lectin-like receptor subfamily C, member 1	Hs.512576	Antigen processing and presentation
HLA-DPA1	Major histocompatibility complex, class II, DP alpha 1	Hs.347270	Antigen processing and presentation Type I diabetes mellitus Cell adhesion molecules (CAMs)
ITGA6	Integrin, alpha 6	Hs.133397	Cell adhesion molecules (CAMs)
GZMB	Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	Hs.1051	Type I diabetes mellitus

HLA-A	Major histocompatibility complex, class I, A	Hs.652059	Antigen processing and presentation Cell adhesion molecules (CAMs) Type I diabetes mellitus
GUCY1B3	Guanylate cyclase 1, soluble, beta 3	Hs.77890	Long-term depression
FASLG	Fas ligand (TNF superfamily, member 6)	Hs.2007	Type I diabetes mellitus
HLA-DMA	Major histocompatibility complex, class II, DM alpha	Hs.351279	Cell adhesion molecules (CAMs)
KIR2DS2	Killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 2	Hs.512572	Antigen processing and presentation
HLA-DOA	Major histocompatibility complex, class II, DO alpha	Hs.631991	Type I diabetes mellitus Antigen processing and presentation Cell adhesion molecules (CAMs)
PTPRM	Protein tyrosine phosphatase, receptor type, M	Hs.49774	Cell adhesion molecules (CAMs)
HLA-DRB1	Major histocompatibility complex, class II, DR beta 1	Hs.701691	Type I diabetes mellitus Antigen processing and presentation Cell adhesion molecules (CAMs)
CD274	CD274 molecule	Hs.521989	Cell adhesion molecules (CAMs)
ASRGL1	Asparaginase like 1	Hs.535326	Cyanoamino acid metabolism
PPP2R2B	Protein phosphatase 2 (formerly 2A), regulatory subunit B, beta isoform	Hs.655213	Long-term depression
MPO	Myeloperoxidase	Hs.458272	Methane metabolism
High expression in TCRαβ+ T-ALL patients			
PRSS2	Protease, serine, 2 (trypsin 2)	Hs.622865	Neuroactive ligand-receptor interaction
TNFSF4	Tumor necrosis factor (ligand) superfamily, member 4 (tax-transcriptionally activated glycoprotein 1, 34kDa)	Hs.181097	Cytokine-cytokine receptor interaction
AREG	Amphiregulin (schwannoma-derived growth factor)	Hs.270833	ErbB signaling pathway
CACNA1H	Calcium channel, voltage-dependent, T type, alpha 1H subunit	Hs.459642	MAPK signaling pathway

TUBB6	Tubulin, beta 6	Hs.193491	Gap junction
HSPA1A	Heat shock 70kDa protein 1A	Hs.520028	MAPK signalling pathway
EDG1	Endothelial differentiation, sphingolipid G-protein-coupled receptor, 1	Hs.154210	Neuroactive ligand-receptor interaction
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	Hs.654408	MAPK signaling pathway
CCR4	Chemokine (C-C motif) receptor 4	Hs.184926	Cytokine-cytokine receptor interaction
IFNB1	Interferon, beta 1, fibroblast	Hs.93177	Cytokine-cytokine receptor interaction Jak-STAT signaling pathway
CCL25	Chemokine (C-C motif) ligand 25	Hs.310511	Cytokine-cytokine receptor interaction
INSR	Insulin receptor	Hs.465744	Type II diabetes mellitus
FPR1	Formyl peptide receptor 1	Hs.753	Neuroactive ligand-receptor interaction
CISH	Cytokine inducible SH2-containing protein	Hs.655334	Jak-STAT signaling pathway
PLCB4	Phospholipase C, beta 4	Hs.472101	Gap junction
RAB23	RAB23, member RAS oncogene family	Hs.555016	Hedgehog signaling pathway

[a]The genes were selected on the basis of statistical significance between two subgroups having $p<0.05$

5.6 Validation of gene expression by Real Time PCR

Based on degree of expression, fold regulation and involvement within signalling pathways (mapped by Natural Language Processing), 7 genes (*NFKB1, FASLG, HLA-DQB1, IFNB1, RXRA, CISH* and *SOCS2*) were shortlisted for further validation by qRT PCR. For six genes, qRT PCR data correlated very well with the microarray data (Figure 5.6). It was observed that *IFNβ1* gene expression remain unchanged between two subgroups. Interestingly, mRNA expression of *RXRA* was found to be reversed between two subgroups as compared to microarray data. Out of these 7 genes, *FASLG, HLA-DQB1, SOCS2* were upregulated in TCRγδ+T-ALL (Figure 5.6). Significant difference was observed in expression of *NFKB1* and *FASLG* between the two subgroups of T-ALL patients.

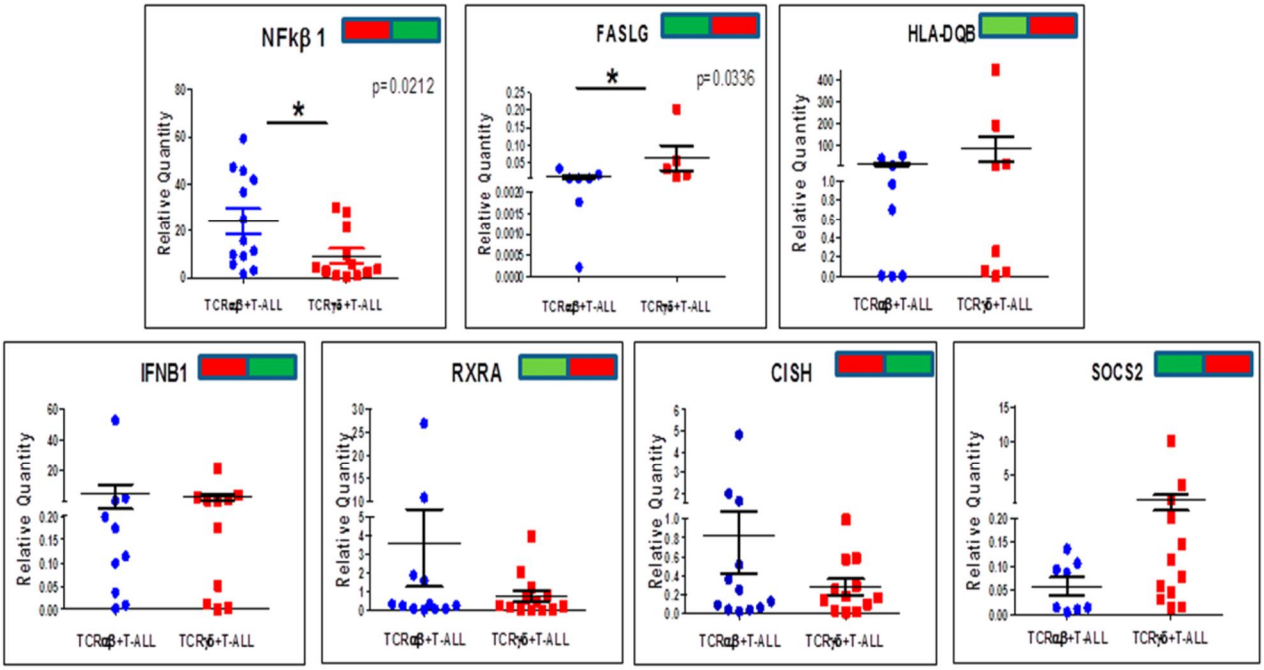


Figure 5.6 qRT based validation of gene expression data in TCRαβ+T-ALL and TCRγδ+ T-ALL patients. Microarray data was validated for 7 genes using real time PCR technique. Genes such as NFKB1, IFNB1, RXRA, CISH were found to be expressed higher in TCRαβ+T-ALL patients (n=13); while FASLG, HLA-DQB1 and SOCS2 were found to be expressed higher in TCRγδ+T-ALL patients (n=17). The level of expression of these genes was consistent with microarray data. Upper right box in each panel indicates gene expression where red represents the high level of expression and green represents the low level. p value provided in the panel indicates statistical significance.

5.7 Comparative analysis of gene mutation in subgroups of T-ALL patients

Few important genes (*CDKN2A, CDKN2B, P53, NOTCH1, IKZF3* and *BRAF*) involved in survival/ chemoresistance and whose gene expression levels were unaltered in TCRγδ+T-ALL and TCRαβ+T-ALL subgroups in microarray analysis were selected to be further evaluated for differences in mutation or CNV.

2D amplitude plots were used to set threshold to determine presence or absence of mutations (Figure 5.7).

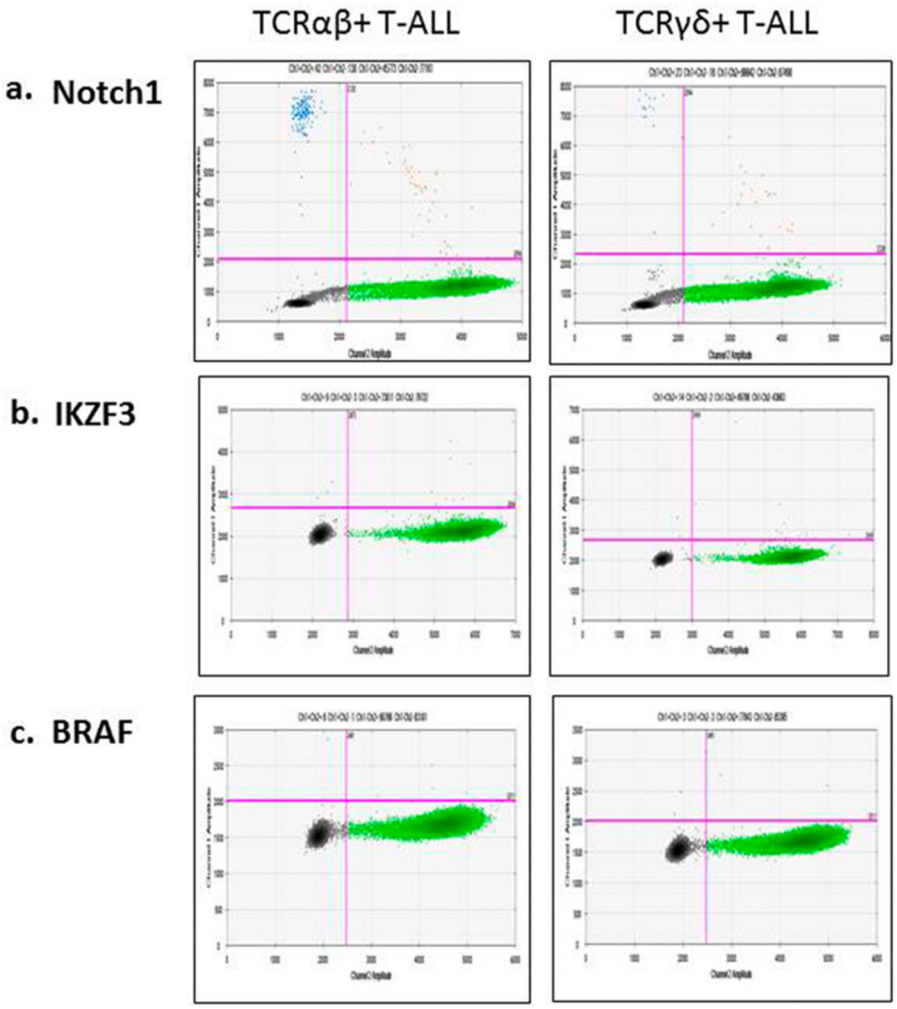


Figure 5.7- 2D amplitude plots for mutation detection. The dot plots represent 2D Amplitude plots for mutation detection in Notch (a), IKZF3 (b) and BRAF (c) in TCRαβ+T-ALL as compared to TCRγδ+T-ALL patients. The plots were generated using QuantaSoft Software (Bio-Rad).

TCRγδ+T-ALL patients exhibited variable frequency of mutations in *NOTCH1* and *IKZF3* as against TCRαβ+T-ALL patients, while BRAF mutations were detected at same level in both the subgroups (Figure 5.8 A-C). DNA of all healthy individuals served as negative control and exhibited no mutation in any of the three genes studied.

Notch mutation was found to be higher in TCRαβ+T-ALL as compared to TCRγδ+T-ALL patients. 17 out of 26 (65.38%) TCRαβ+T-ALL patients showed mutations in Notch as compared to 13 out of 23 (56.52%) TCRγδ+T-ALL patients (Figure 5.8 A).

Mutation in *IKZF3* gene was detected at a low concentration (<0.5 mutant copies in a background of wild type DNA) in 11 of 22 (50%) TCRαβ+T-ALL patients and 8 of 19 (42.10%) TCRγδ+T-ALL patients (Figure 5.8 B). *IKZF3* mutation was found to be high in TCRαβ+T-ALL patients as compared to TCRγδ+T-ALL patients.

Concentration of BRAF mutation was also detected at very low level (<0.25 mutant copies) and number of patients showing the presence of mutation was equal in both the subgroups. 7 of 23 (30.43%) TCRαβ+T-ALL patients and 6 of 19 (31.57%) TCRγδ+T-ALL patients showed the presence of *BRAF* mutation (Figure 5.8 C).

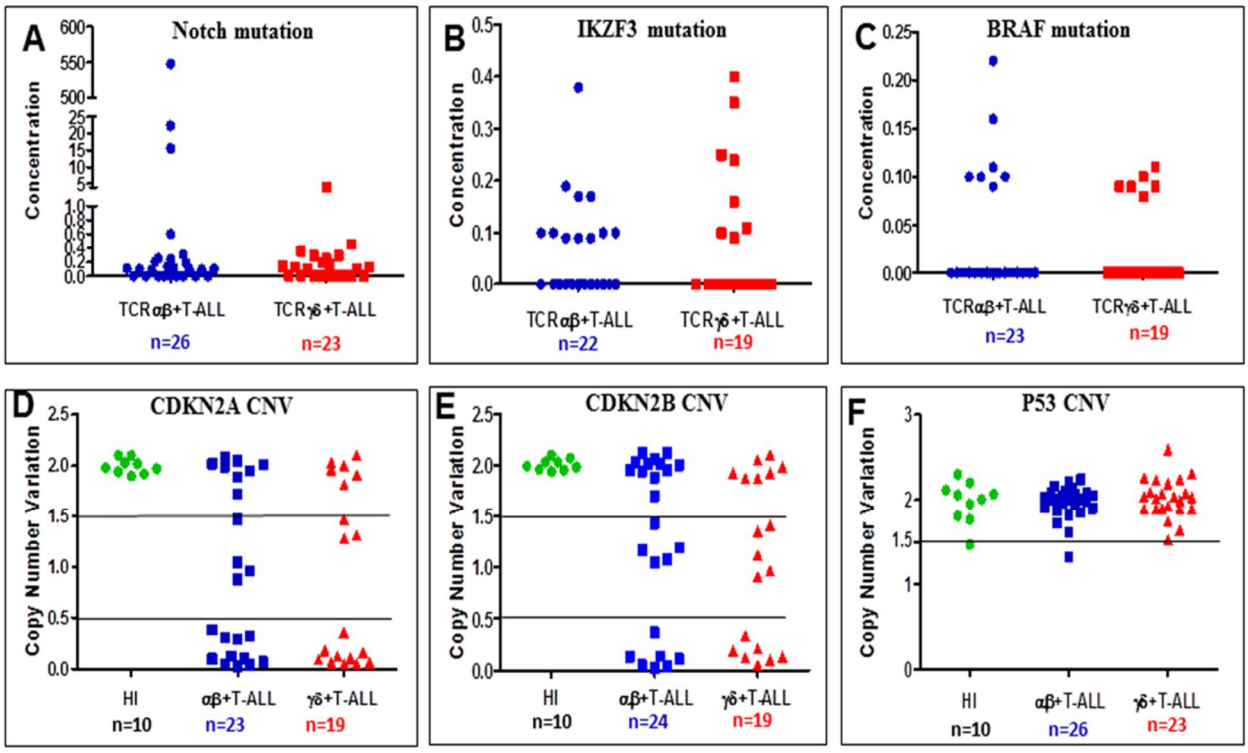


Figure 5.8 Copy number variation and mutation analysis in T-ALL patients. The scatter plots provide gene mutation analysis (panel A, B and C) and comparative CNV (panel D, E and F) in T-ALL patients. Panel A-C: comparative gene mutation analysis of Notch (A), IKZF3 (B) and BRAF (C) in TCRαβ+T-ALL as compared to TCRγδ+T-ALL patients. Panel D-F: comparative CNV of CDKN2A (D), CDKN2B (E) and P53 (F) in TCRαβ+T-ALL, TCRγδ+T-ALL and healthy individuals (HI). CNV range in each panel indicates

homozygous deletion (CNV of 0-0.5), heterozygous deletion (CNV of 0.5-1.5) and wild type copies (CNV of 1.5-2.5). n values provided at bottom of each panel indicate number of patients used.

5.8 Comparative analysis of gene copy number variations in subgroups of T-ALL patients

ddPCR CNV data of *CDKN2A, CDKN2B* and *p53* genes demonstrated distinct signatures of copy numbers in TCRγδ+T-ALL patients as against TCRαβ+T-ALL patients. DNA of healthy individuals served as negative control and exhibited wild type copy numbers of all the three genes (Figure 5.8 D-F).

In TCRαβ+T-ALL patients 17.39% (4 of 23) showed heterozygous deletion and 47.82% (11 of 23) showed homozygous deletion of *CDKN2A* (total deletion 65.21%, Figure 5.8 D). In TCRγδ+T-ALL patients 15.78% (3 of 19) showed heterozygous deletion and 52.63% (10 of 19) showed homozygous deletion of *CDKN2A* (total deletion 68.42%, Figure 5.8 D)

As shown in Figure 5.8 E, 50% TCRαβ+T-ALL patients showed deletion of *CDKN2B* copies, out of which 20.8% (5 of 24) showed heterozygous deletion and 7 of 24 (29.16%) showed homozygous deletion of *CDKN2B*. 63.15% TCRγδ+T-ALL patients showed deletion of *CDKN2B* copies, out of which 26.31% (5 of 19) showed heterozygous deletion and 36.84% (7 of 19) showed homozygous deletion of *CDKN2B*.

P53 copies were found to be deleted in only 1 patient out of 26 TCRαβ+T-ALL patients while there was no deletion of *P53* in any TCRγδ+T-ALL patient (Figure 5.8 F).

5.9 Correlation of mutation status with survival in T-ALL patients

Pairwise comparison of *BRAF, IKZF3* and *NOTCH1* mutation status and TCR clonality of T-ALL patients demonstrated differential survival of the two subgroups of patients viz. TCRαβ+T-ALL and TCRγδ+T-ALL. Based on presence or absence of gene mutation in

NOTCH1, *IKZF3* and *BRAF*, the T-ALL patients (TCRαβ+ and TCRγδ+) showed differences in DFS (Figure 5.9). Although distinct trends were observed in survival, these were not statistically significant (P=0.294, 0.509, and 0.156 for *NOTCH1*, *IKZF3* and *BRAF* respectively).

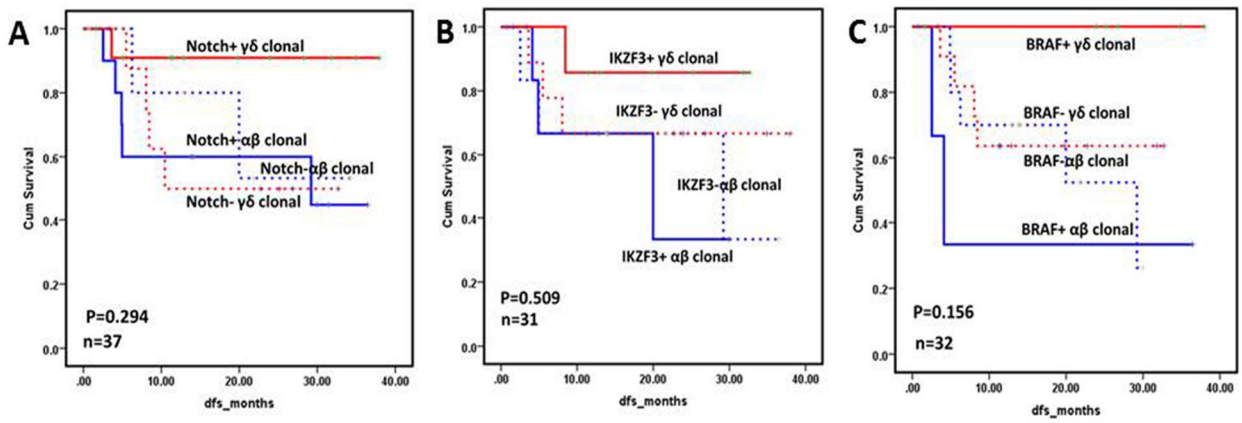


Figure 5.9 Kaplan-Meier survival curves for T-ALL patients based on clonality status and mutations. The panels represent Kaplan-Meier survival curves for DFS of T-ALL subgroups. The parameters considered were T cell clonality and Notch mutation (A); IKZF3 mutation (B); BRAF mutation (C). P value provided in the panel indicates statistical significance and n indicates total number of patients included.

5.10 Correlation of CNV with survival in T-ALL patients

Pairwise comparisons of CDKN2A and CDKN2B CNV and TCR clonality in T-ALL patients (TCRαβ and TCRγδ) were performed. Correlation of DFS with *CDKN2A* and *CDKN2B* CNV and TCR clonality was found to be highly statistically significant (p=0.017 and p=0.045, respectively; Figure 5.10 A-B). In both subgroups of T-ALL, patients with wild type/heterozygous copies of CDKN2A/B showed improved disease free survival over patients with homozygous deletion of CDKN2A/B. In concordance with our previous studies [19, 20], TCRαβ+T-ALL patients always showed poor DFS as compared to TCRγδ+T-ALL patients, even in presence of wild type/heterozygous copies of CDKN2A/B, (Figure 5.10 A-

B). It was observed that *CDKN2A* with wild type/heterozygous deletion in TCRγδ+T-ALL patients showed improved OS over TCRαβ+T-ALL patients but it was not statistically significant (Figure 5.11 A). Similarly, TCRγδ+T-ALL patients with wild type/heterozygous deletion of CDKN2B, showed improved OS over TCRαβ+T-ALL, but was not statistically significant (Figure 5.11 B). In conclusion, TCRγδ+T-ALL subgroup with wild type/heterozygous copies of CDKN2A/B showed significantly (p=0.017 and p=0.045, respectively) improved DFS as compared to TCRαβ+T-ALL.

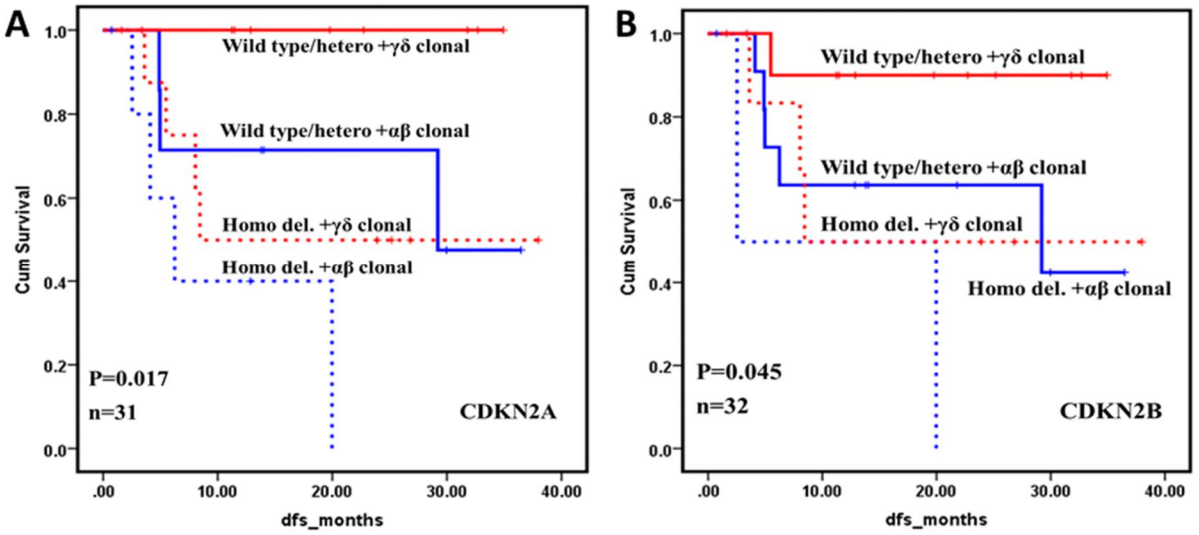


Figure 5.10 Kaplan-Meier survival curves for DFS of T-ALL patients based on clonality status and CNVs. The panels represent Kaplan-Meier survival curves for DFS of T-ALL subgroups. The parameters considered were T cell clonality and CDKN2A CNV (A); and CDKN2B CNV (B). P value provided in the panel indicates statistical significance and n indicates total number of patients included.

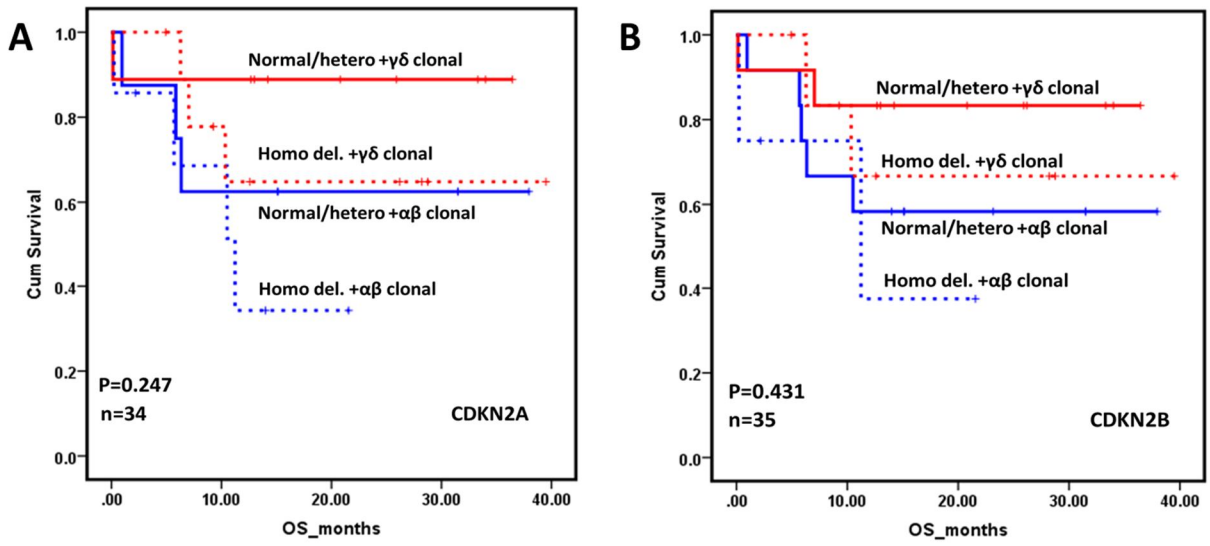


Figure 5.11 Kaplan-Meier survival curves for OS of T-ALL patients based on clonality status and CNVs. The panels represent Kaplan-Meier survival curves for OS of T-ALL subgroups. The parameters considered were T cell clonality and CDKN2A CNV (A); and CDKN2B CNV (B). P value provided in the panel indicates statistical significance and n indicates total number of patients included.

In conclusion, TCRγδ+ T-ALL was identified as a distinct subgroup from TCRαβ+T-ALL using gene expression profiling. Moreover CDKN2A/CDKN2B CNV combined with TCR clonality also emerged as potential prognostic marker in T-ALL patients.

CHAPTER 6

Results-III

Comparison of immunophenotype and cytotoxicity of γδ T cells in TCR γδ and TCR αβ clonal T-ALL patients

Gene expression, copy number variations (CNV), mutations and survival were studied to delineate TCRγδ+T-cell acute lymphoblastic leukemia (T-ALL) as a distinct subgroup from TCRαβ+T-ALL [209]. TCRγδ+T-ALL patients with wild type/heterozygous deletion of CDKN2A/CDKN2B possess significantly better DFS over TCRαβ+T-ALL patients (p=0.017 and 0.045, respectively) and it was shown for the first time by our group, that TCRγδ clonality and CDKN2A/CDKN2B CNV together can serve as potential prognostic markers in management of T-ALL [209].

We further wanted to study differences in immune functions in T-ALL patients classified as TCRγδ clonal and TCRαβ clonal. Hence we performed functional assays like co-culture experiments, cytotoxicity, immune synapse formation and also studied the immunophenotypic differences in these patients.

6.1 Differences in the immunophenotypic characters of TCRγδ clonal Vs TCRαβ clonal patients

We studied the differences in immune cell composition in TCRγδ and TCRαβ clonal subsets of T-ALL patients using multicolour flow cytometry. Immunophenotype analysis of the T-ALL patients with TCRγδ clonality (n=12) Vs TCRαβ clonality (n=26) was carried out.

a) T cell subsets

TCRγδ+T-ALL patients showed significantly high levels of γδ T cells and NKT cells, in the peripheral blood compared to TCRαβ+T-ALL patients. TCRαβ+T-ALL patients showed significantly high levels of αβ T cells in the peripheral blood compared to TCRγδ+T-ALL patients. There was no significant difference observed in expression of other T cell subsets such as total CD3, CD4 (Th) and CD8 (Tc) T cells within the two T-ALL subsets (Figure 6.1).

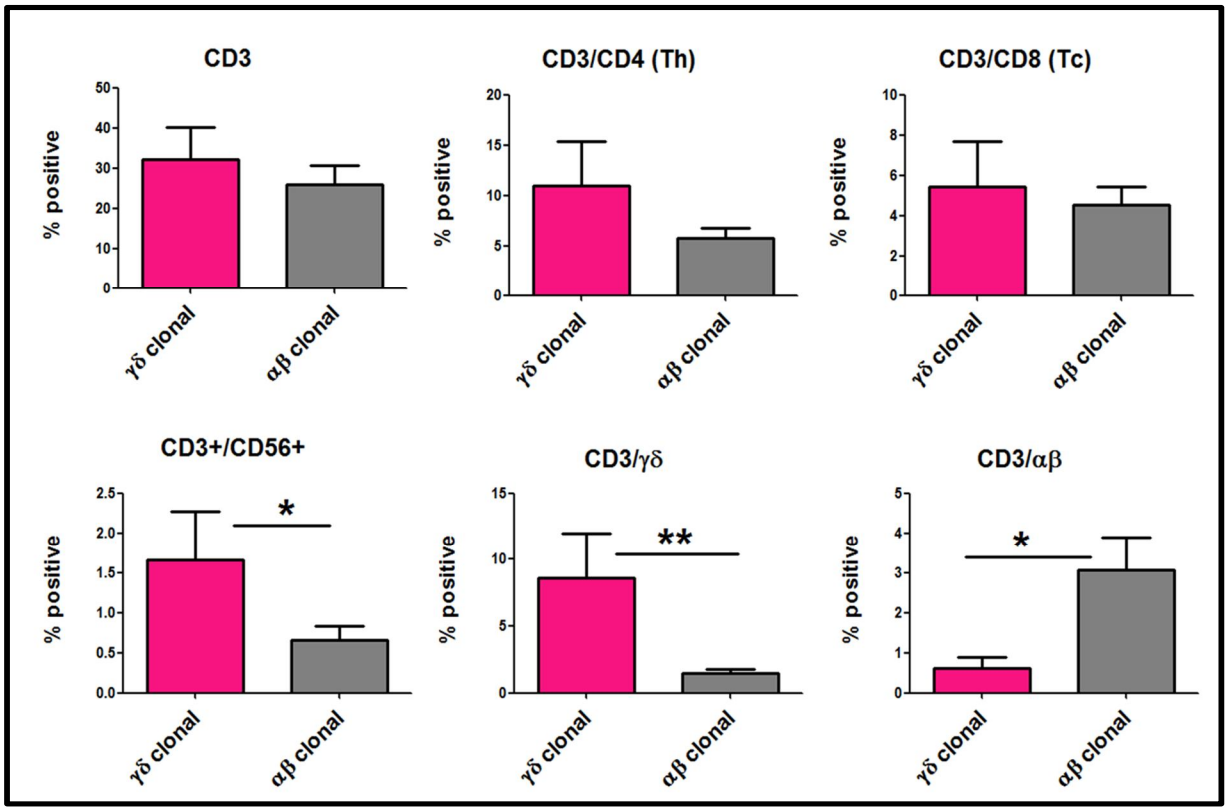


Figure 6.1 Expression of T cell subsets in TCRγδ+T-ALL compared to TCRαβ+T-ALL patients. Figure represents % positive expression of immune cell markers (CD3, CD4, CD8, NKT, γδ T and αβ T cells) in TCRγδ+T-ALL patients (pink bars) compared to TCRαβ+T-ALL patients (grey bars). Data is represented as mean±SE, *: $p<0.05$, **:$p<0.005$.

b) γδ T cell subsets

Vδ2 and Vδ1 T cells, the two major γδ T cell subsets, were compared in TCRγδ+T-ALL and TCRαβ+T-ALL patients. TCRγδ+T-ALL patients showed significantly high levels of Vδ2 T cells in the peripheral blood as compared to TCRαβ+T-ALL patients. Both subgroups show equal expression of Vδ1 T cells, as shown in Figure 6.2.

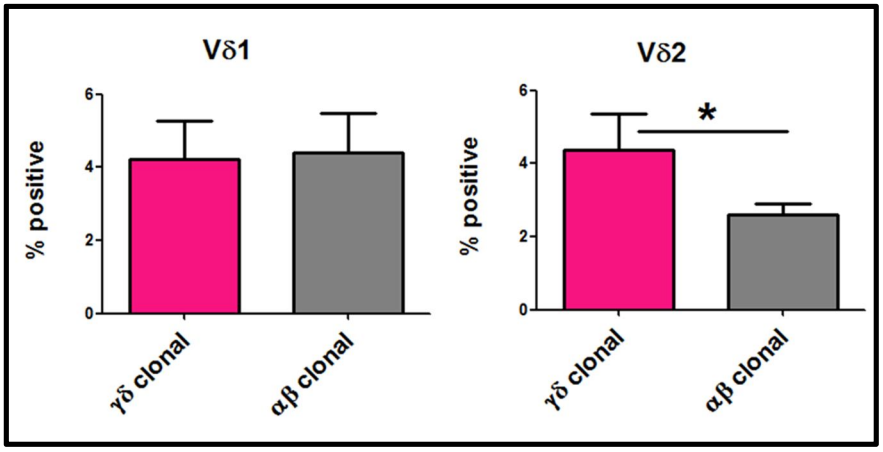


Figure 6.2 Expression of γδ T cell subsets in TCRγδ+T-ALL compared to TCRαβ+T-ALL patients. Figure represents % positive expression of γδ T cell subsets (Vδ1 and Vδ2 T cells) in TCRγδ+T-ALL patients (pink bars) as compared to TCRαβ+T-ALL patients (grey bars). Data is represented as mean±SE, *: $p<0.05$

c) Activation markers

CD3, Vδ2 and Vδ1 T cells were studied for their early and late activation status (CD69 and CD25 respectively) in two subsets of T-ALL patients. As shown in Figure 6.3, TCRγδ+T-ALL patients showed significantly high expression of late activation marker (CD25) on CD3, Vδ2 and Vδ1 T cells compared to TCRαβ+T-ALL patients. Early activation marker (CD69) was significantly high on Vδ1 T cells in TCRγδ+T-ALL patients compared to TCRαβ+T-ALL patients and there were no significant differences in both the subgroups in CD69 expression on CD3 and Vδ2.

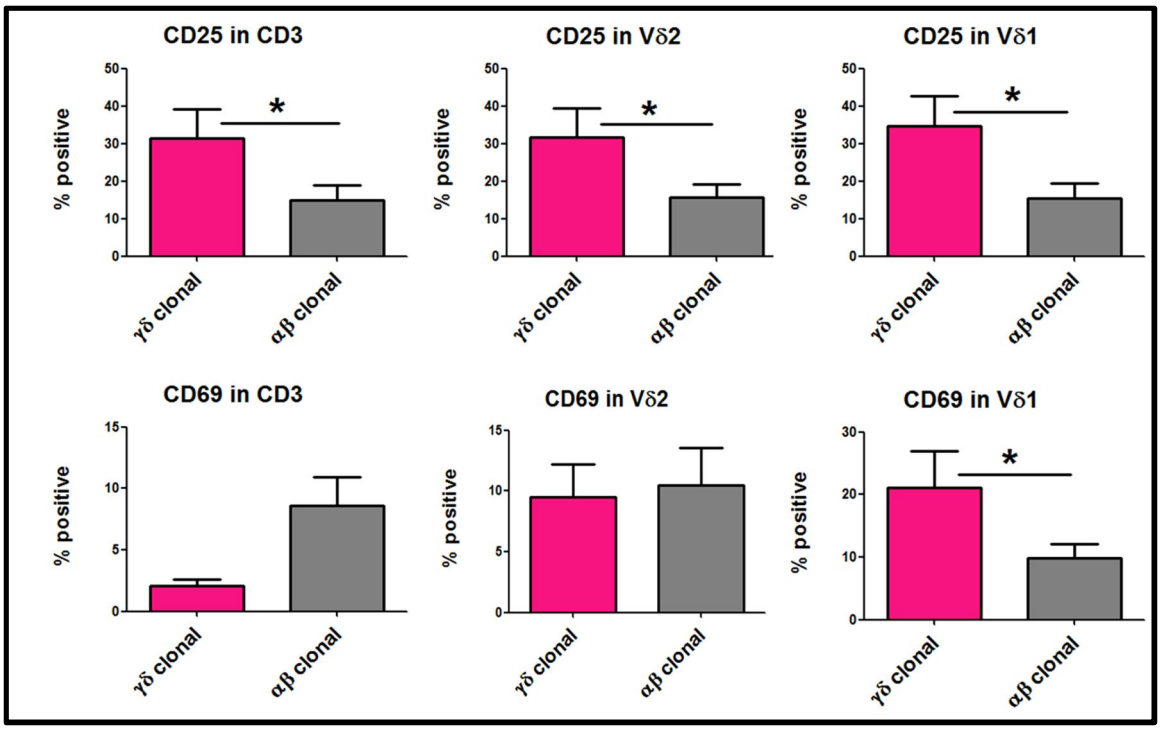


Figure 6.3 Expression of activation markers on CD3, Vδ2 and Vδ1 T cell subsets in T-ALL subgroups. Figure represents % positive expression of activation markers in TCRγδ+T-ALL patients (pink bars) as compared to TCRαβ+T-ALL patients (grey bars). Data is represented as mean±SE, *: $p<0.05$

d) **Memory markers**

Expressions of naive ($CD27^+CD45RA^+$), CM ($CD27^+CD45RA^-$), EM ($CD27^-CD45RA^-$), and TemRA ($CD27^-CD45RA^+$) cells were studied in two subgroups of T-ALL patients. TCRγδ+T-ALL patients show significantly high expression of central memory (CM) and terminally differentiated (TemRA) Vδ1 and Vδ2 T cells compared to TCRαβ+T-ALL patients. Naïve and effector memory (EM) cells are expressed at equal level in CD3, Vδ1 and Vδ2 T cells in both T-ALL subgroups (Figure 6.4).

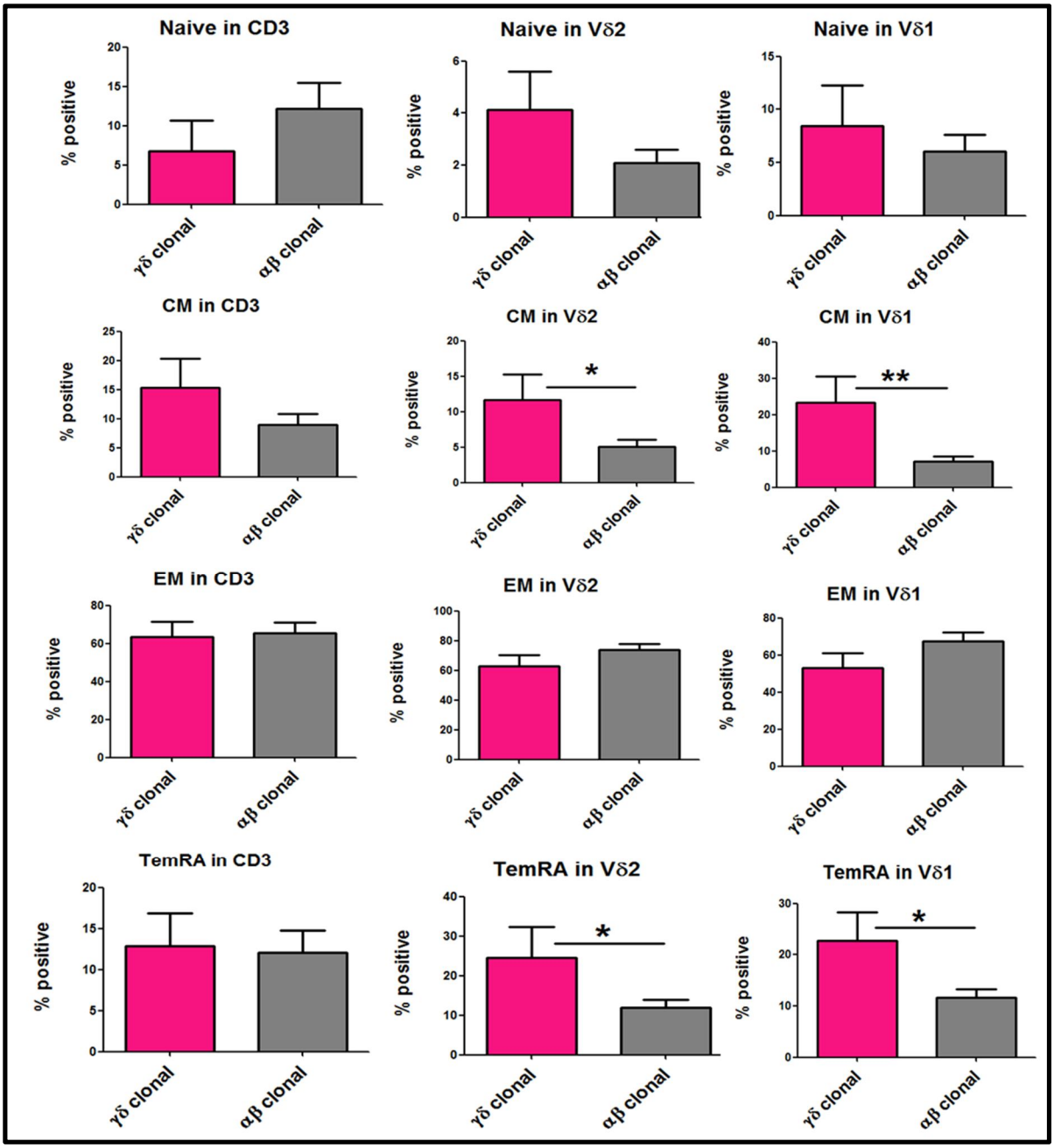


Figure 6.4 Expression of memory markers on CD3, Vδ2 and Vδ1 T cell subsets in T-ALL subgroups. Figure represents % positive expression of memory markers (Naïve, central memory, effector memory, and terminally differentiated) in TCRγδ+T-ALL patients (pink bars) as compared to TCRαβ+T-ALL patients (grey bars). Data is represented as mean±SE, *: $p<0.05$, **:$p<0.005$

As the CM and TemRA cells are significantly high in TCRγδ+T-ALL patients, their immune cells have more potential to proliferate and show effector functions as compared to that of TCRαβ+T-ALL patients.

e) NK cell markers

NKG2D (Natural killer group 2, member D) and CD16b (FCγIIIR) receptor expressions were studied in two subgroups of T-ALL patients. Patients with TCR γδ clonality show significantly high number of NKG2D positive Vδ1 and Vδ2 T cells as compared to patients with TCR αβ clonality. CD16b expression was observed to be significantly higher on CD3 T cells in TCRαβ+T-ALL patients compared to TCR γδ clonal T-ALL patients (Figure 6.5).

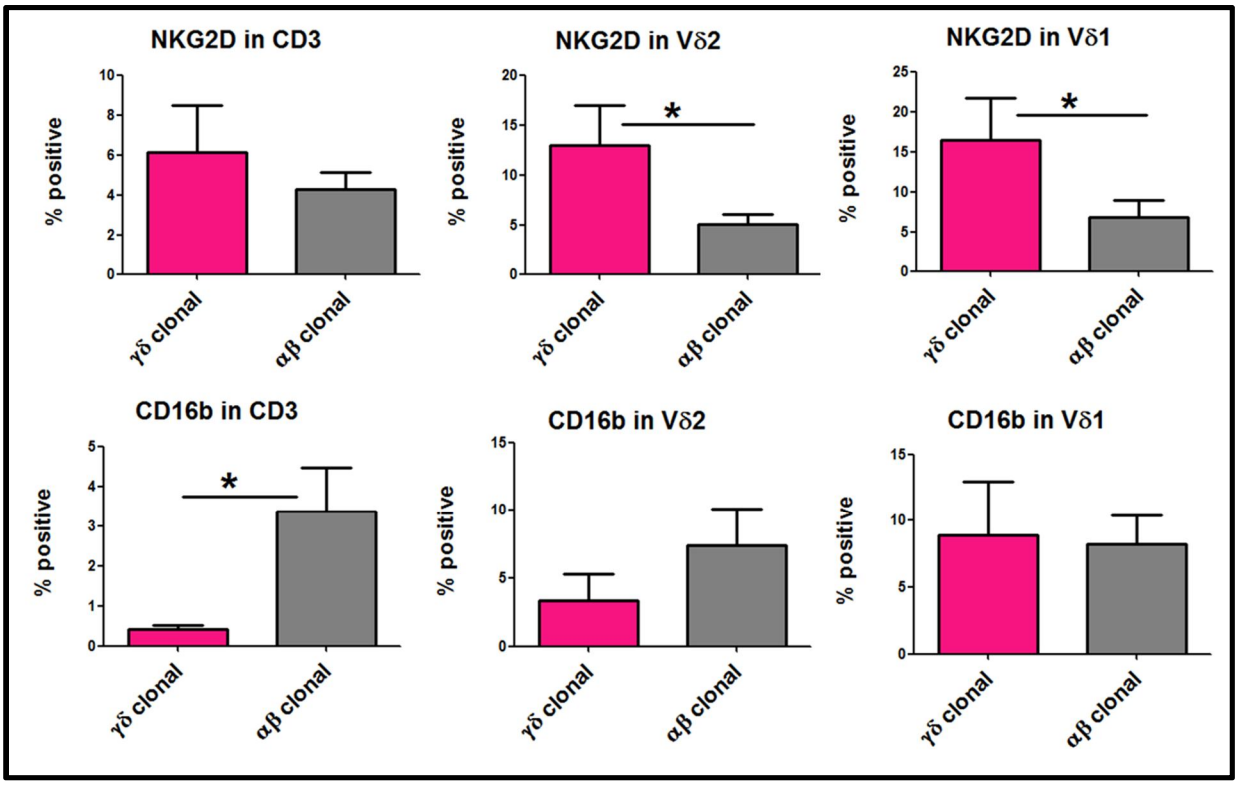


Figure 6.5 Comparison of NKG2D and CD16b expression in TCRαβ+T-ALL and TCRγδ+T-ALL. Figure represents % positive expression of NKG2D and CD16b in TCRγδ+T-ALL patients (pink bars) as compared to TCRαβ+T-ALL patients (grey bars). Data is represented as mean±SE, *: $p<0.05$

f) Perforin expression

We studied the intracellular expression of cytolytic molecule, perforin in two subgroups of T-ALL patients. As shown in Figure 6.6, TCRγδ+T-ALL patients show

significantly high number of Vδ1 and Vδ2 T cells expressing perforin molecules compared to TCRαβ+T-ALL patients.

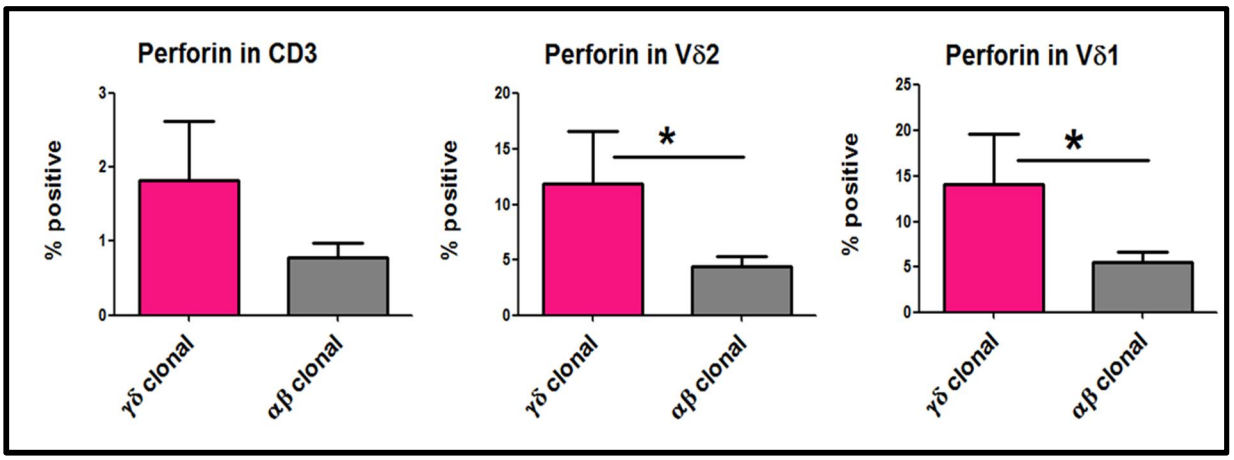


Figure 6.6 Expression of perforin in TCRαβ+T-ALL and TCRγδ+T-ALL patients. Figure represents % positive expression of perforin in TCRγδ+T-ALL patients (pink bars) as compared to TCRαβ+T-ALL patients (grey bars). Data is represented as mean±SE, *: $p<0.05$

6.2 Differences in the cytotoxic potential of γδ T cells against TCRγδ clonal Vs TCRαβ clonal leukemic blasts

a) ^{51}Cr release assay

Our earlier studies have shown selective survival advantage to TCR γδ+T-ALL patients over TCR αβ+T-ALL patients [19, 20]. Cytotoxic potential of purified γδ T lymphocytes isolated from HI, against leukemic blast cells from two T-ALL patient subgroups as a target was studied using Chromium Release Assay. TCRαβ or TCRγδ clonal leukemic blasts were treated with Zoledronate for 16-18 hrs or kept untreated, as described in chapter 3. Higher % cytotoxicity of γδ T cells was observed against Zoledronate treated (25%) or untreated (8.07%) TCR γδ clonal leukemic blasts (n=3) as compared to cytotoxicity observed against Zoledronate treated (15%) or untreated (2.16%) TCR αβ clonal leukemic blasts (n=3) at 1:10 target: effector ratio (Figure 6.7). Although the clear trend was noted, differences in % cytotoxicity were not significant between the two

subgroups of T-ALL patients due to limited sample size. TCR γδ clonal leukemic blasts may be more susceptible to lysis by effector γδ T cells over TCR αβ clonal leukemic blasts.

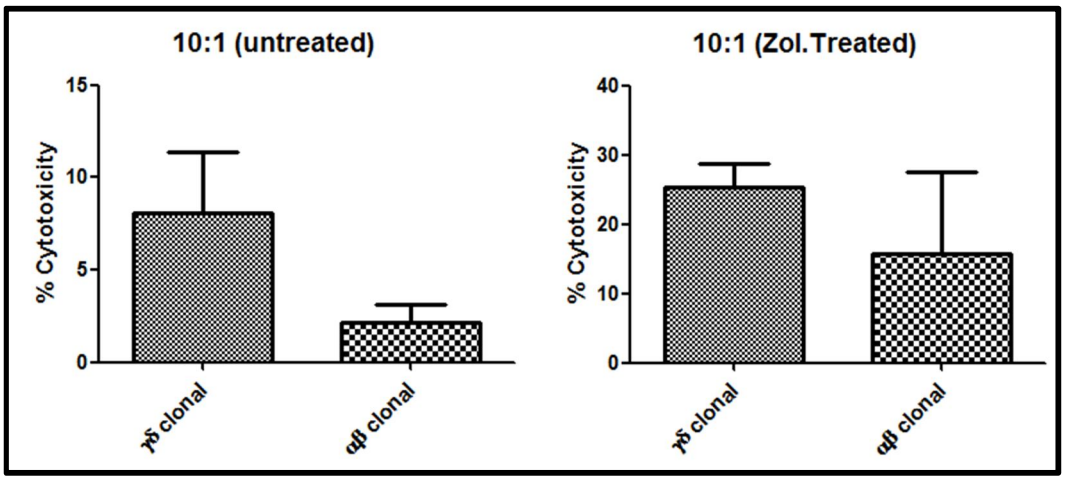


Figure 6.7 Cytotoxic potential of γδ T cells against TCRαβ and γδ clonal leukemic blasts. % cytotoxicity of γδ T cells against leukemic blast cells is measured using ^{51}Cr release assay. γδ T cells were co-cultured with ^{51}Cr labelled leukemic blasts at 10:1 ratio. X-axis indicates TCR clonality of T-ALL patients. Data is represented as mean±SE

b) Perforin release at immune synapse

Cytotoxic T lymphocytes like γδ T cells secrete perforin and granzyme to kill the tumor targets. We studied the release of perforin molecules by γδ T cells when they are co-cultured with untreated or Zoledronate treated leukemic blasts of TCRαβ or TCRγδ clonal leukemic patients. It was observed that effector γδ T cells showed increased release of perforin at the immune synapse with TCR γδ clonal leukemic blasts as compared to that of with αβ clonal leukemic blasts after Zoledronate treatment. There was no difference in perforin release by γδ when they are co-cultured with untreated TCR γδ clonal/ αβ clonal leukemic blasts (Figure 6.8).

This indicates better immune synapse formation of γδ T cells with TCRγδ clonal leukemic blasts and increased killing as compared to TCRαβ clonal leukemic blasts.

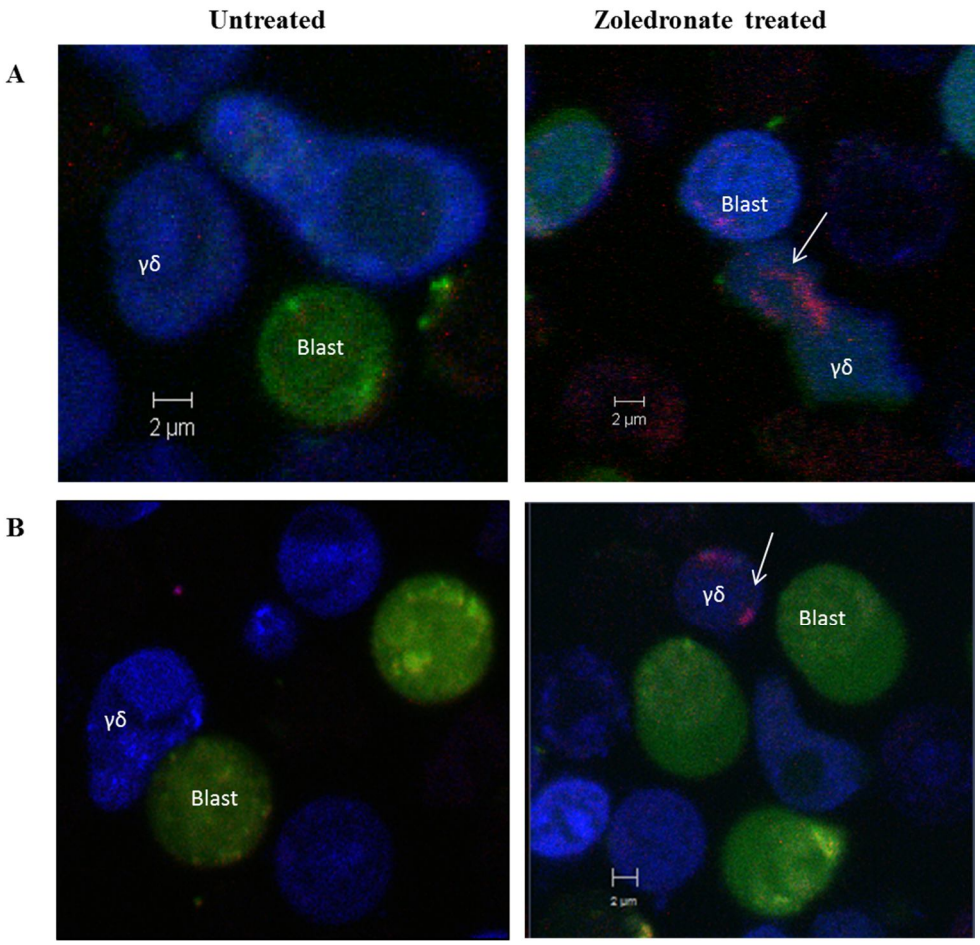


Figure 6.8 Release of perforin at the immune synapse between γδ T cells and TCRαβ /γδ clonal leukemic blasts. Untreated and Zoledronate treated leukemic blasts (Calcein AM, green) were co-cultured with γδ T cells (CMAC blue, Perforin, Lysotracker red) at 1:2 ratio. Perforin molecules were stained with specialised dye, Lysotracker red. Release of perforin at immune synapse was studied using live cell imaging on LSM780 confocal microscope. A. TCR γδ clonal leukemic blasts co-cultured with γδ T cells and B. TCR αβ clonal leukemic blasts co-cultured with γδ T cells. Arrow indicates perforin molecules.

6.3 Expansion of Vδ2 T cells after co-culture of leukemic blasts with healthy PBMCs

Co-culture experiments were performed to study expansion of Vδ2 T cells. TCR γδ clonal leukemic blasts and TCR αβ clonal leukemic blasts were co-cultured with CFSE labelled HI PBMCs for 6 days in presence of rIL-2. PBMCs with rIL-2 alone served as baseline control and PBMCs with rIL2 and BrHPP served as positive control. On day 6, culture was harvested and dual color immunophenotyping was carried out using CFSE/Vδ2 PE to find

Vδ2 T cell proliferation. It was observed that higher percentage of Vδ2 T cells are expanded when HI PBMCs are co-cultured with TCR γδ clonal leukemic blasts (17.8%, Figure 6.9 D) as compared to TCR αβ clonal leukemic blasts (5.45%, Figure 6.9 C). It was interesting to note that co-culture of HI PBMCs with TCR γδ clonal leukemic blasts resulted in expansion of Vδ2 T cell subset (17.8%) that was comparable to that observed after stimulation with BrHPP (15.1%, Figure 6.9, B).

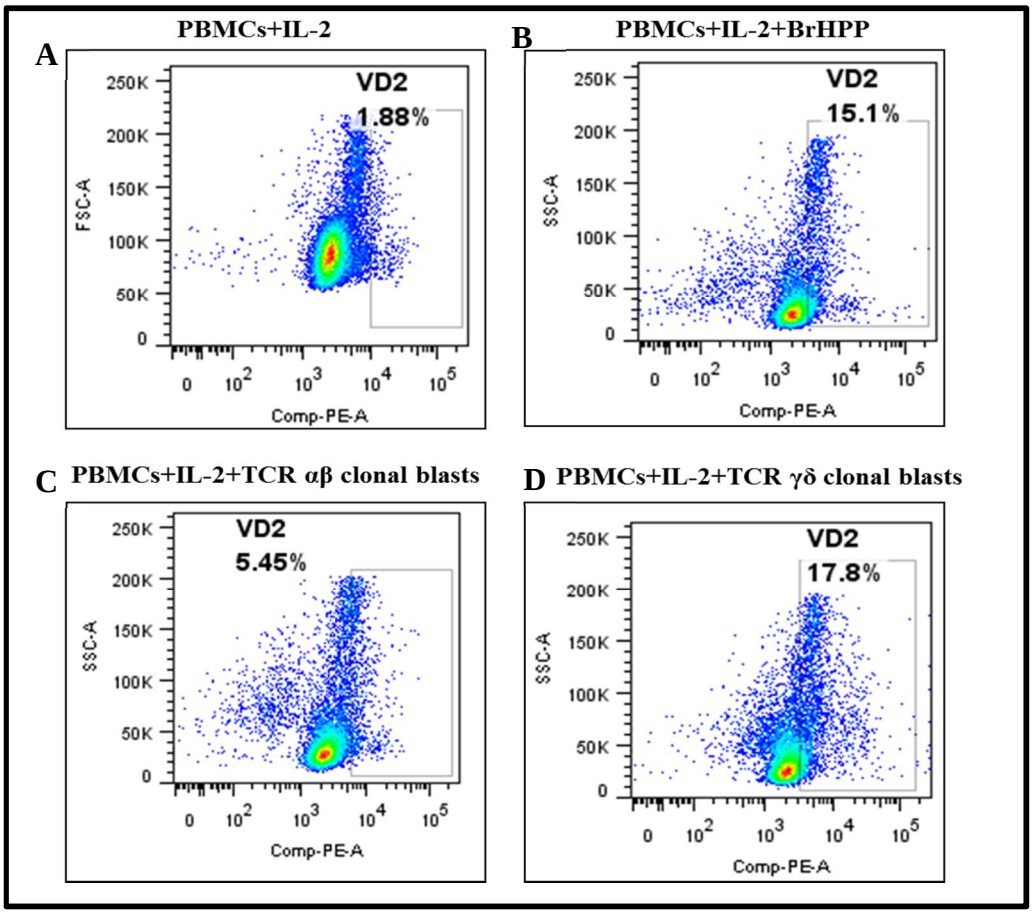


Figure 6.9 Proliferation of Vδ2 T cells in CFSE assay. Proliferation of Vδ2 T cells after co-culture of HI PBMCs with, A. rIL-2 alone, B. rIL-2 and BrHPP, C. rIL-2 and TCR αβ clonal leukemic blasts, D. rIL-2 and TCR γδ clonal leukemic blasts. Numbers in upper right of the panels represent percent divided Vδ2 T cells.

Hence TCR γδ clonal leukemic blasts have ability to stimulate higher number of Vδ2 T cells from HI PBMCs as compared to TCR αβ clonal blasts.

6.4 Differences in the immune synapse formation in TCRγδ clonal and TCRαβ clonal patients with healthy γδ T cells

Immune synapse formation indicates recognition and killing of antigen expressing cell by immune cell. Immune synapse formation between TCRγδ clonal or TCRαβ clonal leukemic blasts and healthy γδ T cells was studied using laser confocal microscopy.

a) F-Actin polarization at the synapse

F-actin polarization is significantly more for immune synapse of γδ T cells with TCR γδ clonal leukemic blasts as compared to TCRαβ clonal leukemic blasts (Figure 6.10, A). Hence TCR γδ clonal leukemic blasts are able to form significantly better immune synapse with effector γδ T cells over TCR αβ clonal leukemic blasts (Figure 6.10, B)

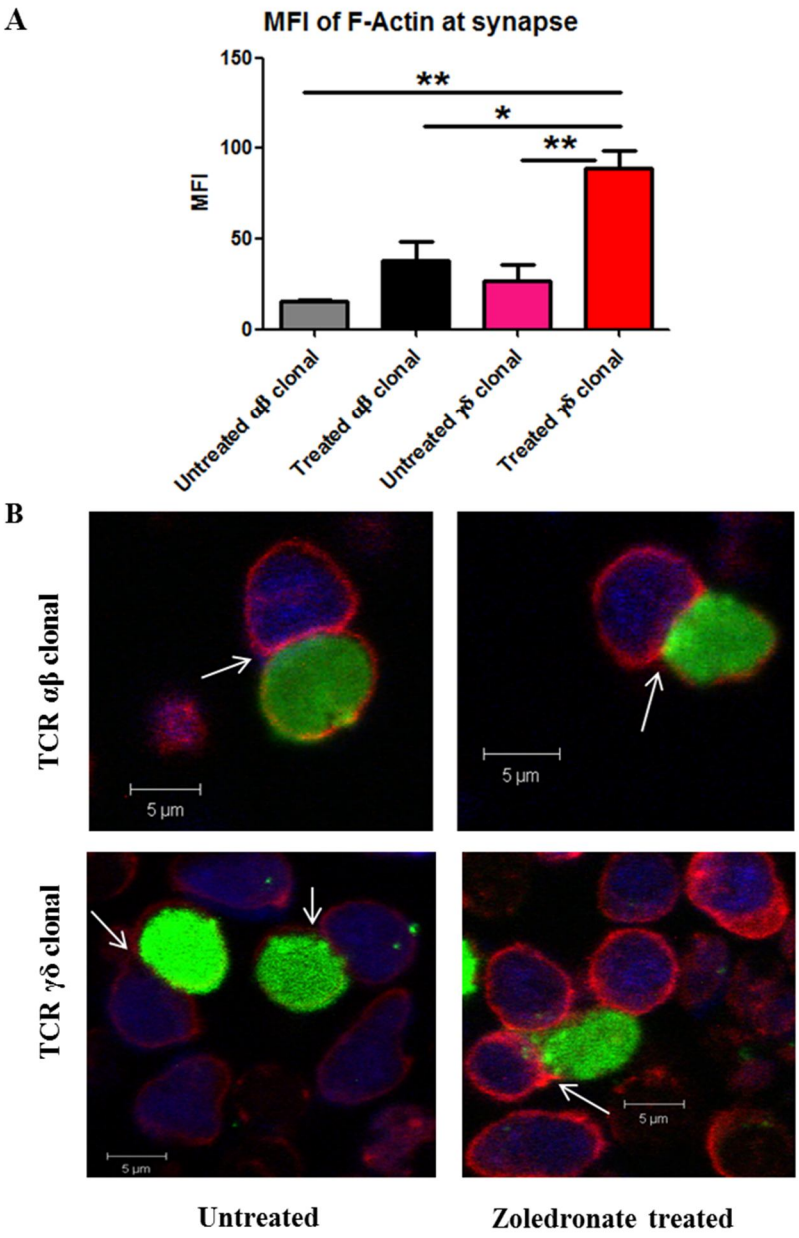


Figure 6.10 F-actin polarization at the immune synapse between γδ T cells and TCRαβ or γδ clonal leukemic blasts. Leukemic blasts (Calcein AM, green) were co-cultured with γδ T cells (CMAC blue) and conjugates were stained for f-actin (phalloidin TRITC, red). **A.** Median fluorescence intensity (MFI) of f-actin at the immune synapse (*: $p<0.05$, **:$p<0.005$). **B.** F-actin polarization (red, indicated with arrow) at the immune synapse between leukemic blasts and γδ T cells. Experiments were performed in triplicates and figure is a representative image of all. Upper panel is for TCRαβ clonal and lower panel is for γδ clonal, left panels are untreated leukemic blasts and right panels for zol.treated blasts.

b) Expression of LFA-1 (CD11a) at the immune synapse between γδ T cells and TCR γδ clonal /αβ clonal leukemic blasts

As shown in figure 6.11, LFA-1 (CD11a) expression was found to be higher at the immune synapse between TCR γδ clonal leukemic blasts and γδ T cells (Panel A). The expression was further increased when TCR γδ clonal leukemic blasts were treated with Zoledronate as compared to Zoledronate treated TCR αβ clonal leukemic blasts (Panel B).

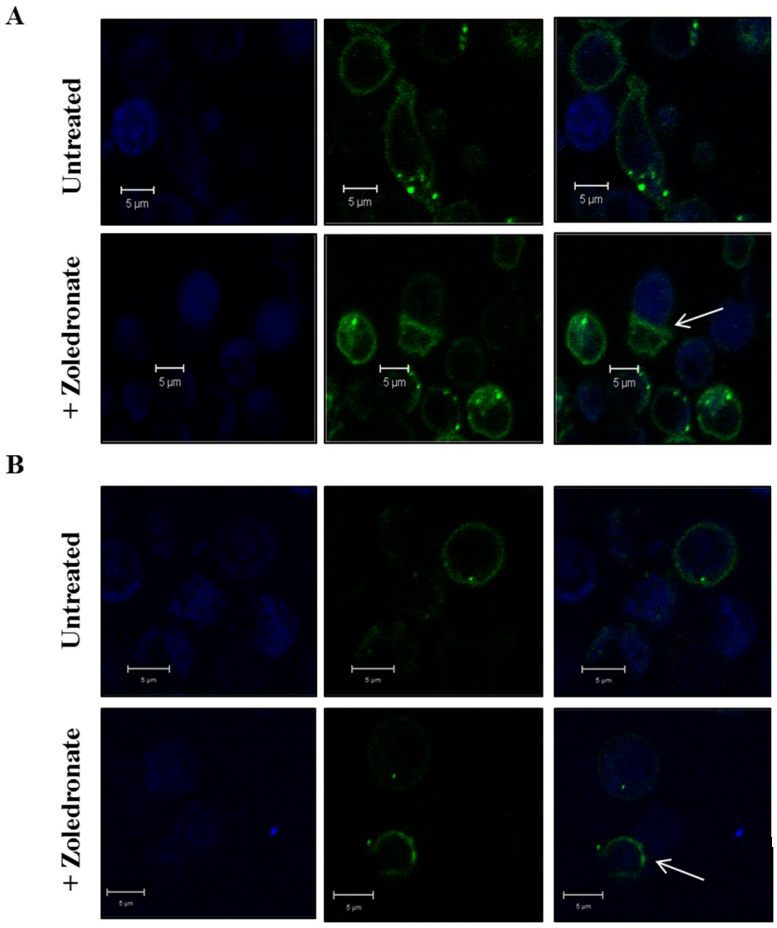


Figure 6.11 Expression of LFA-1 at the immune synapse between γδ T cells and leukemic blasts Leukemic blasts (CMAC blue) untreated or zoledronate treated were co-cultured with γδ T cells (LFA-1 FITC, green). **A**. TCR γδ clonal leukemic blasts **B**. TCR αβ clonal leukemic blasts. Arrow indicates LFA-1 expression at immune synapse.

c) Expression of Dynamin-2 at the immune synapse between γδ T cells and TCR γδ clonal /αβ clonal leukemic blasts

Leukemic blasts were treated with Zoledronate or kept untreated and co-cultured with γδ T cells and dynamin-2 expression was studied at the immune synapse. TCR γδ clonal leukemic blasts do not show difference in Dynamin-2 expression at immune synapse with effector γδ T

cells (Figure 6.12, A) over TCR αβ clonal leukemic blasts after Zoledronate treatment or untreated (Figure 6.12, B).

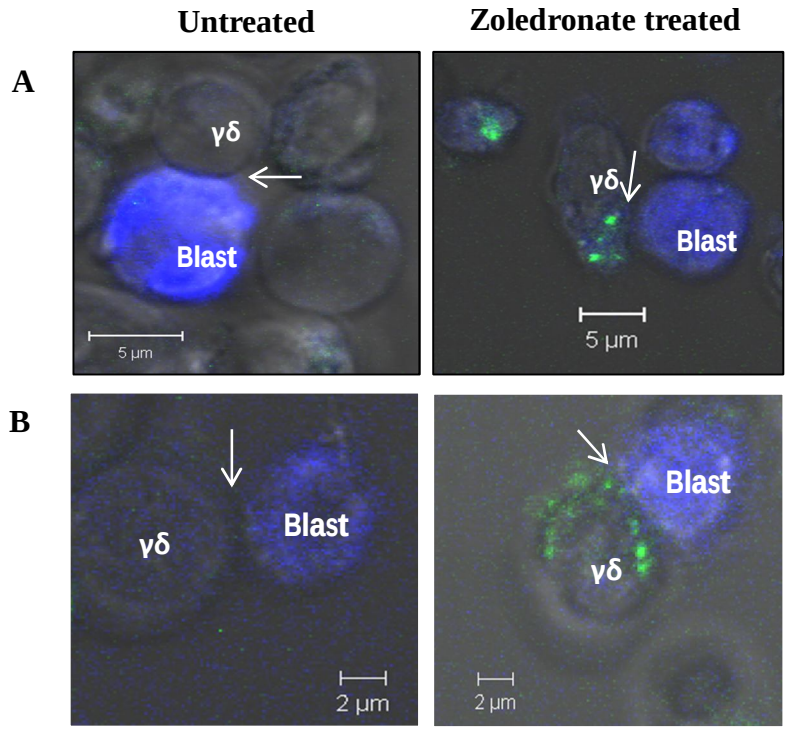


Figure 6.12 Expression of Dynamin-2 at the immune synapse between γδ T cells and TCRαβ or γδ clonal leukemic blasts. Leukemic blasts (CMAC blue) untreated or Zoledronate treated were co-cultured with γδ T cells and stained for Dynamin-2 (green). **A.** TCR γδ clonal leukemic blasts and **B.** TCR αβ clonal leukemic blasts. Arrow indicates Dynamin-2 expression at immune synapse.

d) Expression of CD166 at the immune synapse between γδ T cells and TCR γδ clonal /αβ clonal leukemic blasts

CD166 (also called activated leukocyte-cell adhesion molecule or ALCAM) expression was analysed at the immune synapse. Leukemic blasts were treated with Zoledronate or kept untreated and co-cultured with γδ T cells and CD166 expression was studied at the immune synapse. TCR γδ clonal leukemic blasts show higher expression of CD166 at immune synapse with effector γδ T cells (Figure 6.13, Panel A) over TCR αβ clonal leukemic blasts after Zoledronate treatment or untreated (Figure 6.13, Panel B).

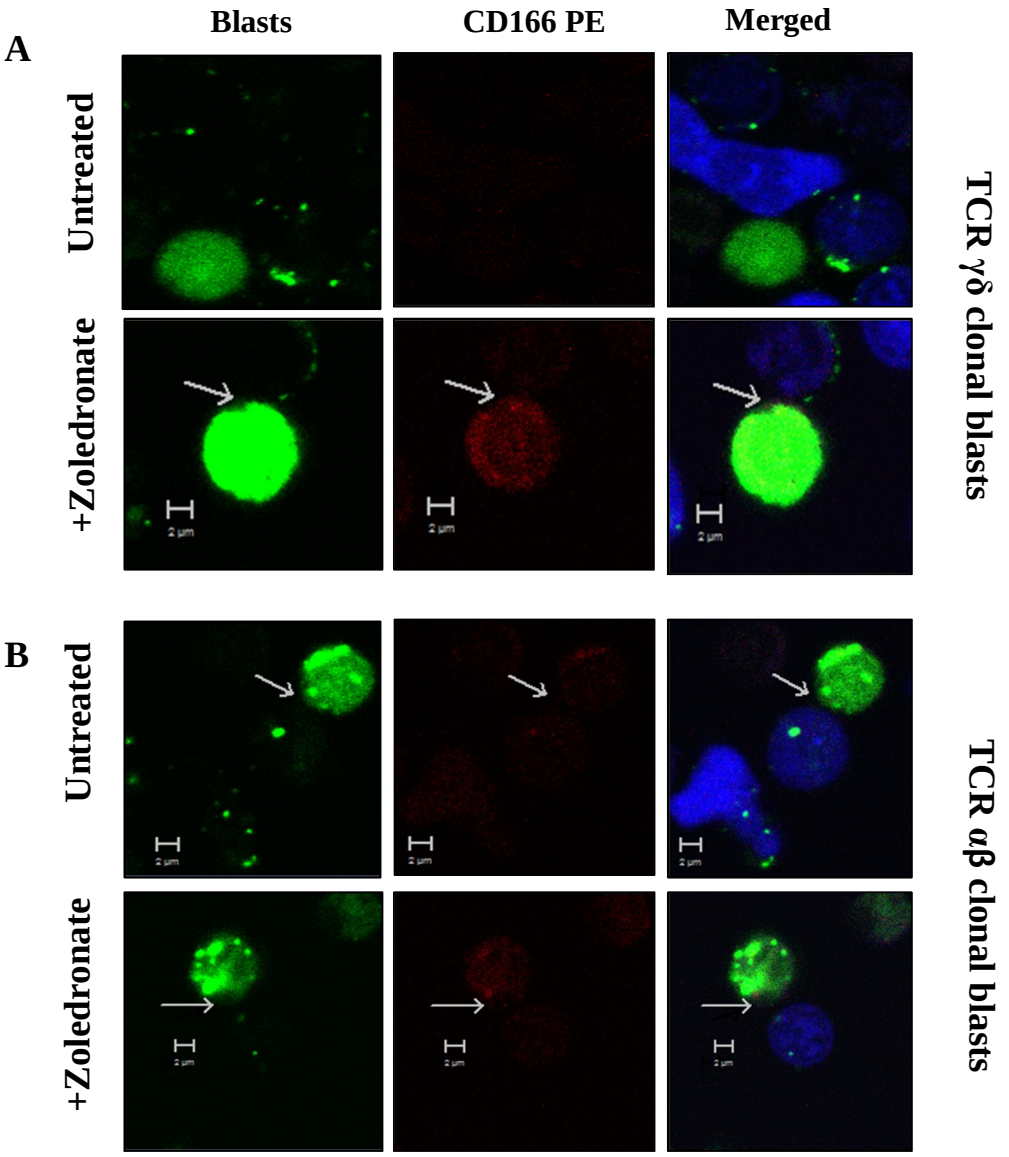


Figure 6.13 Expression of CD166 at the immune synapse between γδ T cells and TCRαβ or γδ clonal leukemic blasts. Leukemic blasts (Calcein AM, green) were co-cultured with γδ T cells (CMAC blue) and conjugates were stained for CD166 (PE, red). **A.** TCR γδ clonal leukemic blasts and **B.** TCR αβ clonal leukemic blasts. Arrow indicates CD166 expression at immune synapse.

In conclusion, TCRγδ+ T-ALL patients show distinct immunophenotype from TCRαβ+T-ALL patients. TCR γδ clonal leukemic blasts are more susceptible to lysis by γδ T cells, as these blasts form better immune synapse and are able to expand more Vδ2 T cells as compared to TCR αβ clonal leukemic blasts. The underlying functional differences in the immune cells and leukemic blasts explain the survival advantage of TCRγδ+ T-ALL patients.

CHAPTER 7

Results-IV

Role of γδ T cells in Acute Lymphoblastic Leukemia patients receiving allogeneic BMT

Over the last 4 decades, allogeneic hematopoietic stem cell transplantation (allo-HSCT) from an HLA-matched donor, either related or unrelated, has been increasingly used to treat patients affected by several malignant or non-malignant hematological disorders [210]. HSCT is associated with several potentially lethal complications, for example, relapse of the malignant disease, graft rejection, infectious complications, and GVHD, a condition where donor graft cells attack healthy host tissue [211]. It is reported that patients who develop increased numbers of donor derived circulating γδ T cells following haploidentical or partially mismatched HSCT experienced a significant leukemia-free survival (LFS) and overall survival (OS) advantage and fewer infections [31, 48, 195]. Most studies focus on αβ T cells and the impact of γδ T cell reconstitution after HSCT has not been well investigated. Since γδ T cells are known to have protective roles during various types of infections, it was interesting to evaluate the impact of γδ T cell reconstitution on infections like CMV post BMT. γδ T cells may provide an ideal source of T cell immunotherapy for leukemia, as they exhibit biologic characteristics consistent with innate immune recognition, thereby allowing them to respond to malignancy without recognition of alloantigens that could result in GvHD [31].

In the present chapter immunophenotypic analysis of γδ T cells along with other immune effector cells was carried out in ALL patients undergoing allogeneic BMT. Immunophenotyping was performed at different time intervals pre and post BMT. Immune reconstitution post BMT was correlated with incidence of GvHD, relapse, CMV reactivation, treatment related mortality and WBC reconstitution.

7.1 Patient characteristics

Peripheral blood was collected from patients undergoing allogeneic BMT (n=17) at various time points viz., at baseline, post BMT day 0, day 30, day 90, day 180 and day 365. We also collected respective donor's blood sample at baseline. Out of 17 ALL patients analysed, 3

patients were T-ALL and 14 patients were positive for B-ALL. Median age of recipients and donors was <25 years. Majority of recipients and donors were males. 7 patients showed complete remission till day 365, 7 patients relapsed before day 365, and 9 patients died post BMT. 6 patients had acute Graft versus Host Disease (GvHD) and 5 patients had chronic GvHD. Acute GvHD was observed at three sites gut, liver and skin whereas chronic GvHD was observed at 4 sites, eye, liver, skin and oral. 12 patients showed CMV reactivation post BMT. Details of patient characteristics are given in table 7.1

Table 7.1: Characteristics of patients undergoing allogeneic bone marrow transplantation

Total patients	**n=17**
Diagnosis	B-ALL: 14 T-ALL: 3
Median Age	Recipients: 19 (3-39)***** Donors: 22 (3-48)
Sex (M/F)	Recipients: 16 males /1 female Donors: 11 males /6 females
Median presenting WBC count ($x10^6$/L)	14000 (1200-297600)
Stage at BMT (Number of cases)	CR1: 10 CR2: 04 CR3: 02 Relapse: 01
Median CD 34 Cells/kg($x10^6$/Kg)	5.02 (2.98-7.38)
WBC engraftment day	15 (13-20)
Platelate engraftment day	14 (11-25)

Acute GvHD (Yes/No)	6/11
Grade	Grade I: 03 Grade II: 01 Grade III: 01 Grade IV: 01
Sites	Gut:05 Skin: 03 Liver: 01
Chronic GvHD (Yes/No)	5/9
sites	Eye: 02 Skin: 03 Oral: 04 Liver: 01
Infections (Yes/No)	CMV reactivation (12/5) Fungal infection (2/15) EBV reactivation (3/14) Haemorrhagic cystitis (2/14)
Treatment related mortality (Yes/No)	3/6
Abbreviations: GvHD, Graft versus Host Disease; CR, Complete remission.* Median range	

7.2 Immune scenario in ALL patients undergoing allogeneic hematopoietic stem cell transplantation

Immunophenotype of patients undergoing allogeneic BMT was carried out at various time points viz., at baseline, post BMT day 0, day 30, day 90, day 180 and day 365. Various immune cell markers were compared at different time points pre and post BMT.

a) T, B and NK cell markers in patients pre and post BMT.

Patients undergoing BMT showed decreasing number of CD3, CD4, CD8, NK, NKT and CD19 cells on day 0 of BMT. As the recipient is given conditioning chemotherapy before BMT, immune cell population has depleted. CD3, CD8, NK, NKT and CD19 cells showed steady increase post BMT till day 365. There was no change in the number of CD4 cells post BMT as compared to day 0. CD8, NK and CD19 cells showed significant increase on day 365 post BMT as compared to day 0.

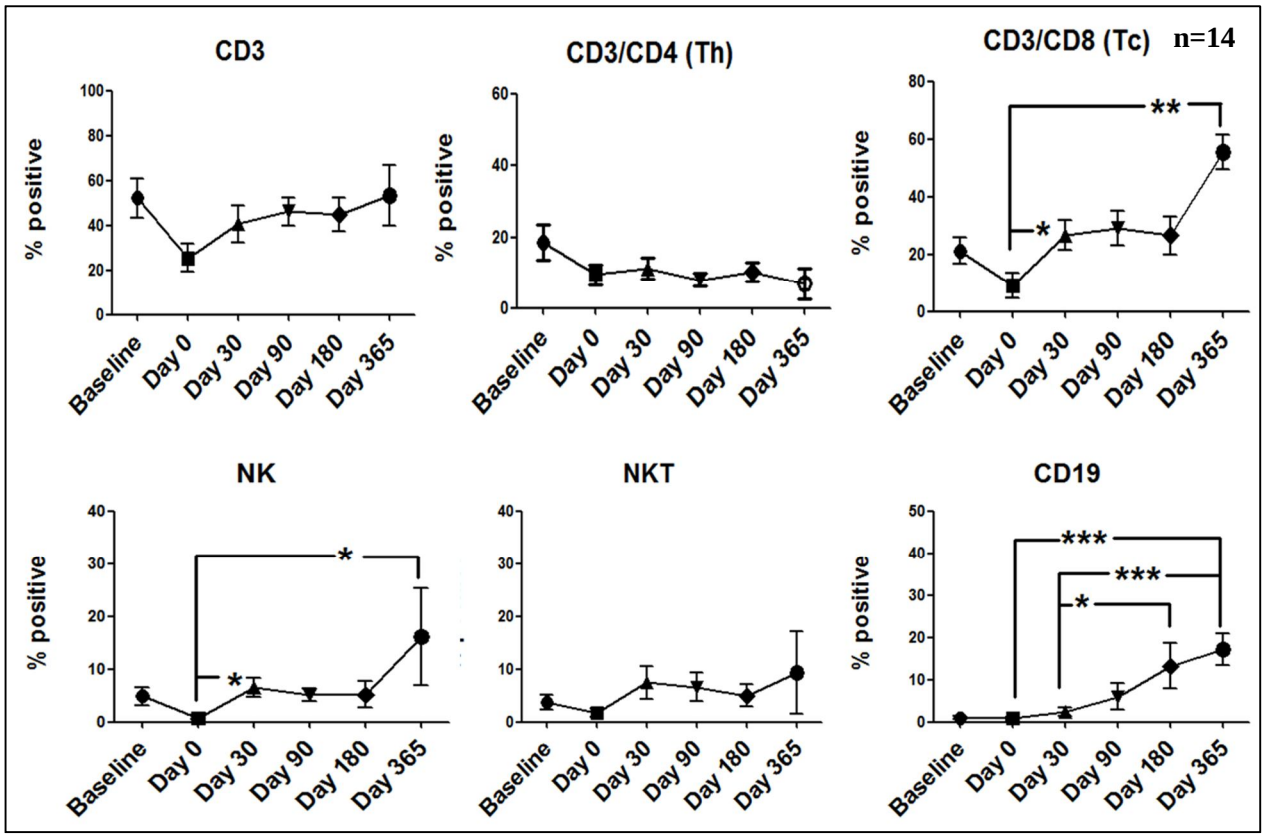


Figure 7.1: Comparison of immune cell subsets in patients pre and post BMT. Patients undergoing BMT were studied before and after BMT at different time intervals. X-axis indicates BMT timeline and Y-axis indicates % positive expression of immune cell markers. Data is represented as mean±SE, *: $p<0.05$, **:$p<0.005$, ***:$p<0.0005$.

b) γδ T cell subsets

Recipients of allogeneic BMT expressed similar levels of Vδ1 and Vδ2 T cell subsets pre and post BMT. γδ T cells were decreased on day 0 and showed significant increase on day 180 post BMT. On day 365 post BMT, the γδ T cells remained similar to baseline levels.

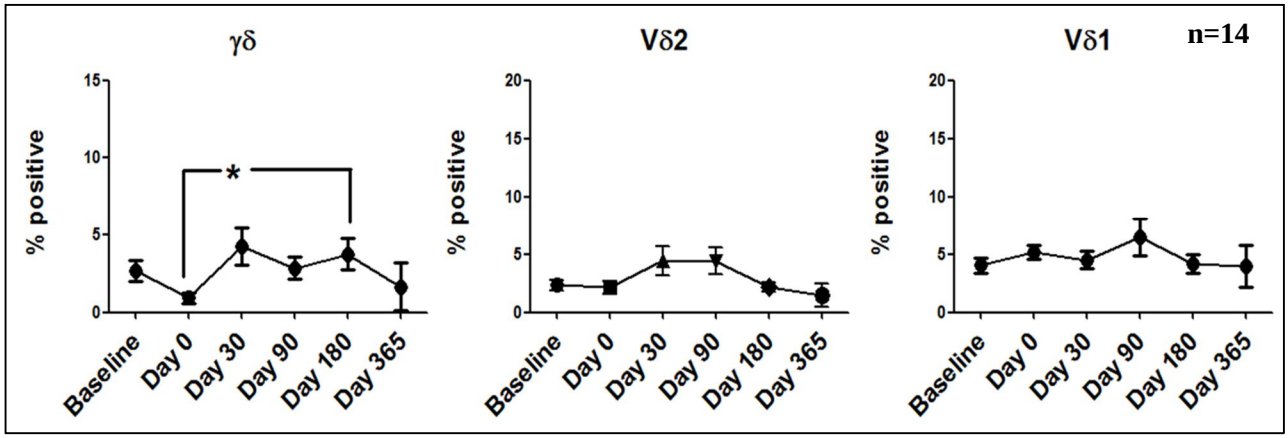


Figure 7.2: Comparison of γδ T cell subsets in patients pre and post BMT. Patients undergoing BMT were studied before and after BMT at different time intervals. X-axis indicates BMT timeline and Y-axis indicates % positive expression of immune cell markers. Data is represented as mean±SE, *: $p<0.05$

7.3 Comparison of immune scenario between ALL patients achieving remission and relapsed/ dead patients after receiving allogeneic hematopoietic stem cell transplantation

Recipients undergoing allogeneic BMT were studied for immune cell composition pre and post BMT till day 365. At the end of sample collection, patients were divided in two categories viz., those who achieved remission and those who relapsed or died post BMT. Out of 14 patients studied for immunophenotype, 7 patients achieved remission and 7 patients relapsed or died post BMT.

a) T, B and NK cell markers in patients at baseline and after BMT

As shown in Figure 7.3, patients who are in remission show differential level of immune cell marker expression as compared to patients who relapsed or died. Patients who achieve remission, had baseline differences in CD4 cells with significantly higher number of CD4 cells as compared to relapsed/dead patients. Patients in remission had higher level of CD3 cells post BMT on day 180, and significantly high CD8 T cells on day 180 post BMT as compared to patients who relapsed/died. Patients in remission also showed significantly high levels of NK, NKT and B cells on day 30 post BMT as compared to patients who relapsed/died. The results indicate patients who achieve remission have different immune cell composition at various time intervals post BMT as compared to relapsed/dead patients.

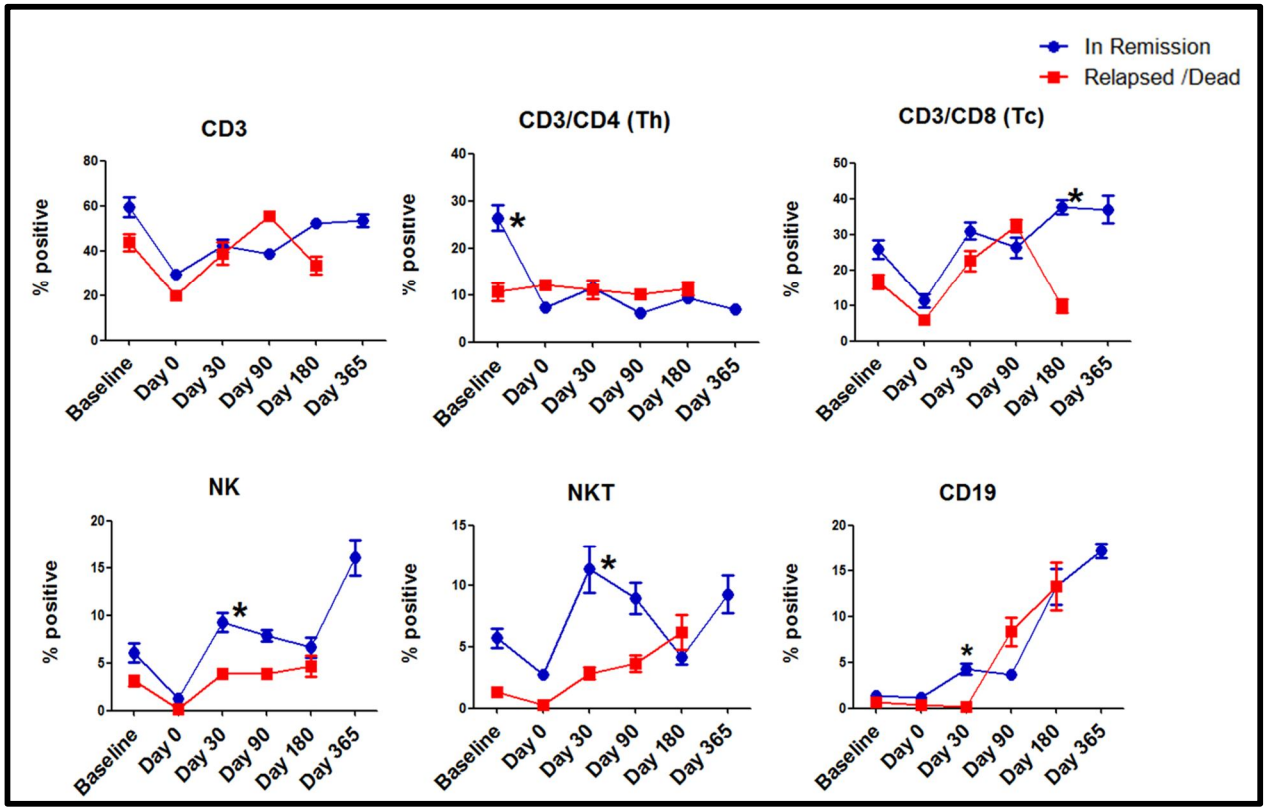


Figure 7.3: Comparison of immune cell subsets pre and post BMT between remission and relapsed/dead patients. Blue line represents patients in remission and red line represents relapsed or dead patients. X-axis indicates BMT timeline and Y-axis indicates % positive expression of immune cell markers. Data is represented as mean±SE, *: $p<0.05$

b) γδ T cell subsets

It was observed that patients who relapsed/died showed an increasing trend in expression of Vδ2 T cells till day 90 post BMT which decreases on day 180. Whereas there was no difference in levels of Vδ1 and γδ T cells between two categories of patients at different time points (Figure 7.4).

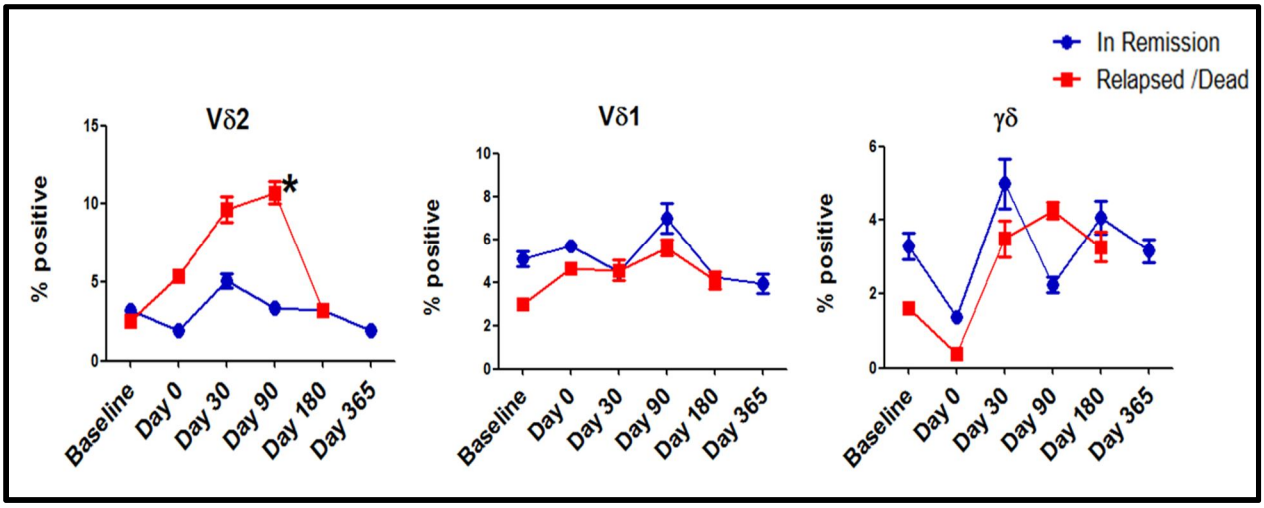


Figure 7.4: Comparison of γδ T cell subsets pre and post BMT between remission and relapsed/dead patients. Blue line represents patients in remission and red line represents relapsed or dead patients. X-axis indicates BMT timeline and Y-axis indicates % positive expression of immune cell markers. Data is represented as mean±SE , *: $p<0.05$

c) Activation markers

Expression of early and late activation markers was studied in the two categories of patients. Patient who achieve remission and who relapsed/died did not show differences in expression of activation markers at baseline. It was observed that patients who are in remission showed significantly higher expression of CD3/CD25 (late activation marker) on day 0 and Vδ2/CD25 on day 180 post BMT. CD69 (early activation) expression was high in the patients who relapsed/died, but it did not show significant differences.

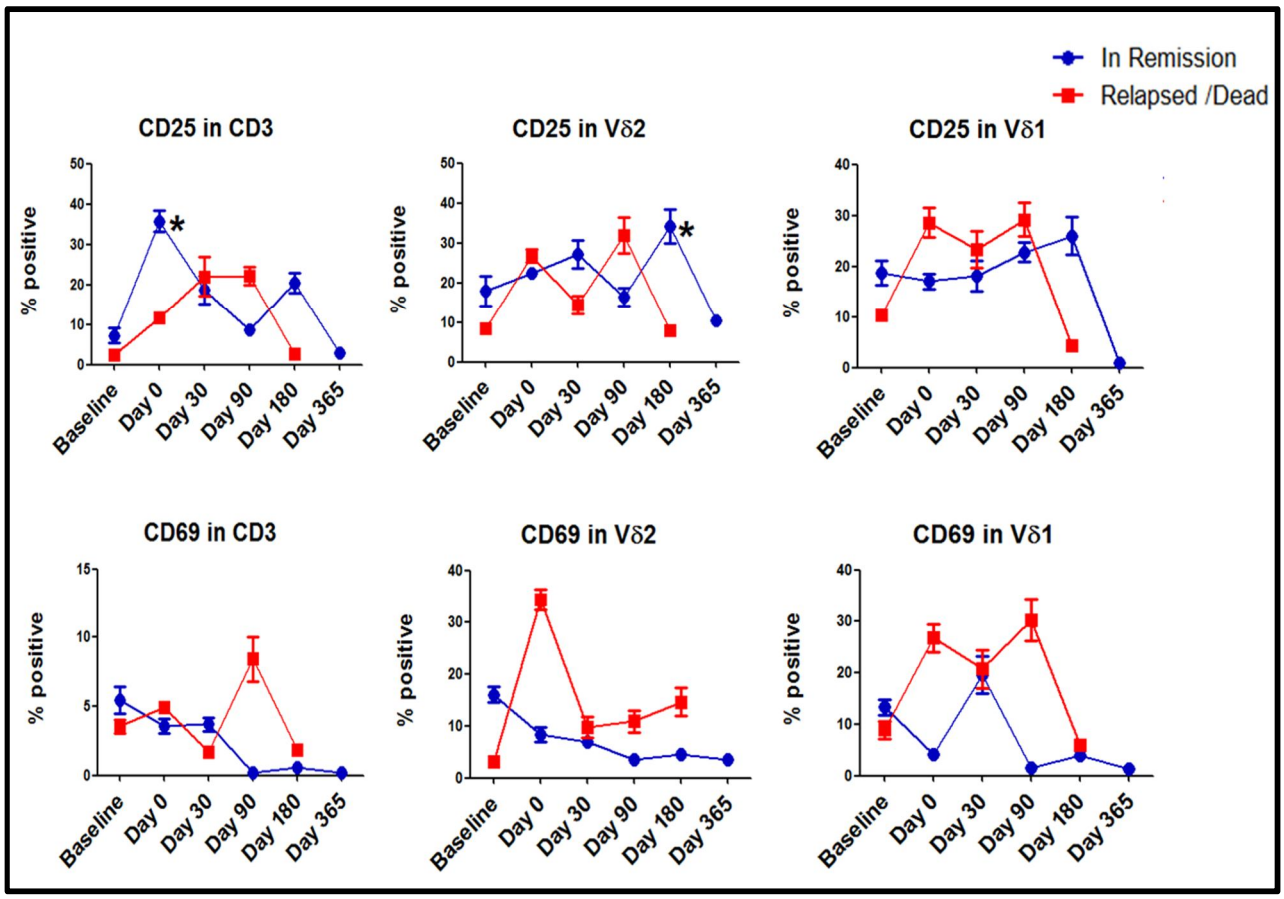


Figure 7.5: Expression of activation markers pre and post BMT in remission and relapsed/dead patients. Blue line represents patients in remission and red line represents relapsed or dead patients. X-axis indicates BMT timeline and Y-axis indicates % positive expression of immune cell markers. Data is represented as mean±SE, *: $p<0.05$

d) Memory markers

Naïve and central memory (CM) cells show higher proliferation capacity whereas effector memory (EM) and terminally differentiated (TemRA) cells show higher functional properties. Patients in remission showed significant higher baseline levels of naïve Vδ2, CD3 TemRA and Vδ1 TemRA T cells as compared to patients who relapsed/died. Naïve cell population decreased subsequently post BMT. Central memory (CM) cells did not show any difference in the trend between two categories of patients and these cells also decreased over the time period post BMT. Effector memory (EM) cells showed increase on day 0 and they were lowest on day 90. CD3 EM cells were significantly high in patients at remission on day 180 as compared to patients who relapsed/died. Terminally differentiated (TemRA) cells

were found to be lowest on day 0 of BMT, and subsequent increase was observed in both the groups of patients (Figure 7.6).

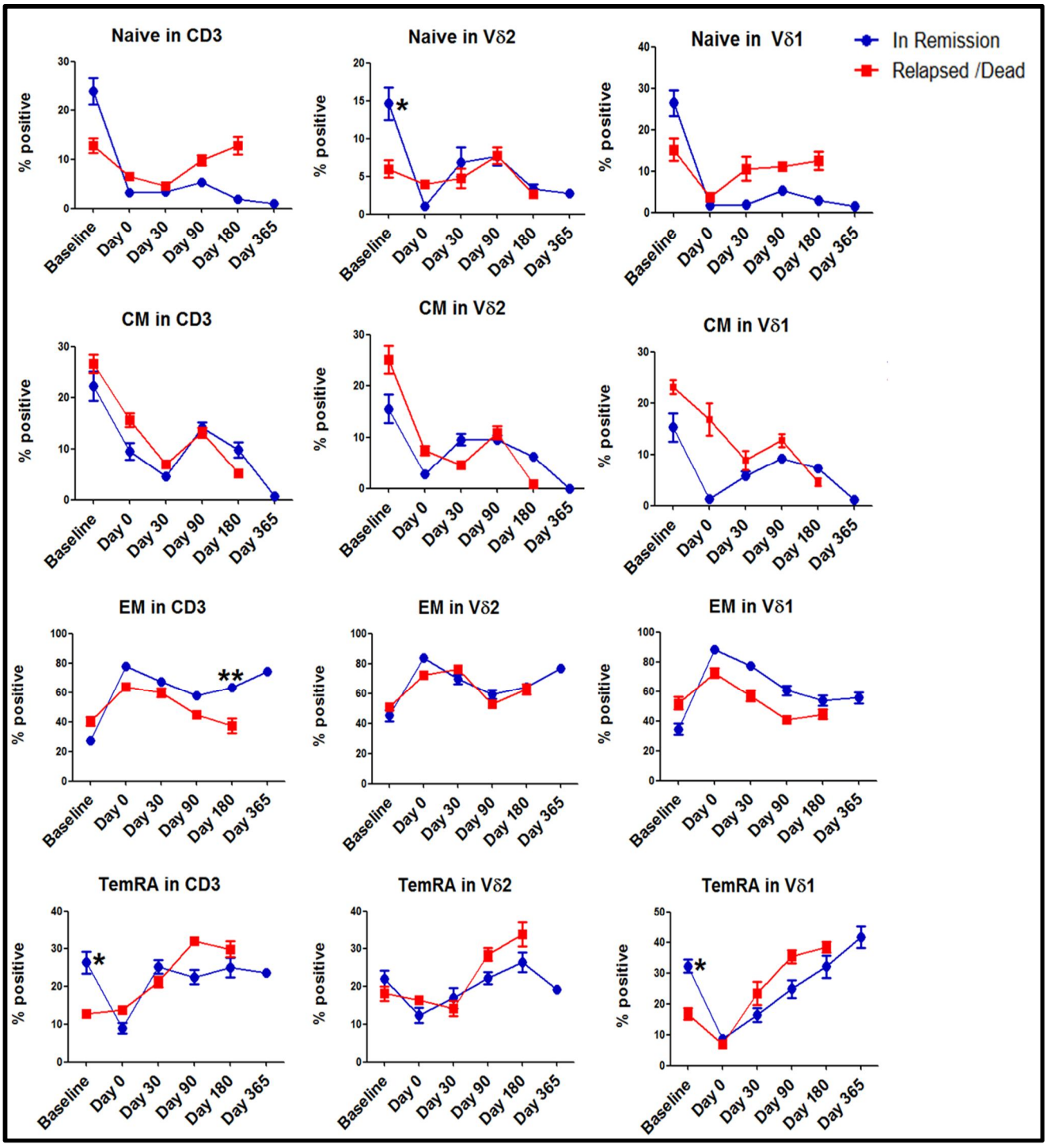


Figure 7.6: Expression of memory markers pre and post BMT in remission and relapsed/dead patients. Blue line represents patients in remission and red line represents relapsed or dead patients. X-axis indicates BMT timeline and Y-axis indicates % positive expression of immune cell markers. Data is represented as mean±SE *: $p<0.05$, **:$p<0.005$.

e) NK cell makers

NKG2D (Natural Killer Group 2D) and CD16b (FcγRIII) expressions were studied in recipients undergoing BMT. Patients who relapsed or died showed significantly high level of NKG2D expression on day 90 post BMT as compared to patients in remission. Whereas no distinct trend was observed for CD16b expression and there was no significant difference in expression between the two categories of patients (Figure 7.7).

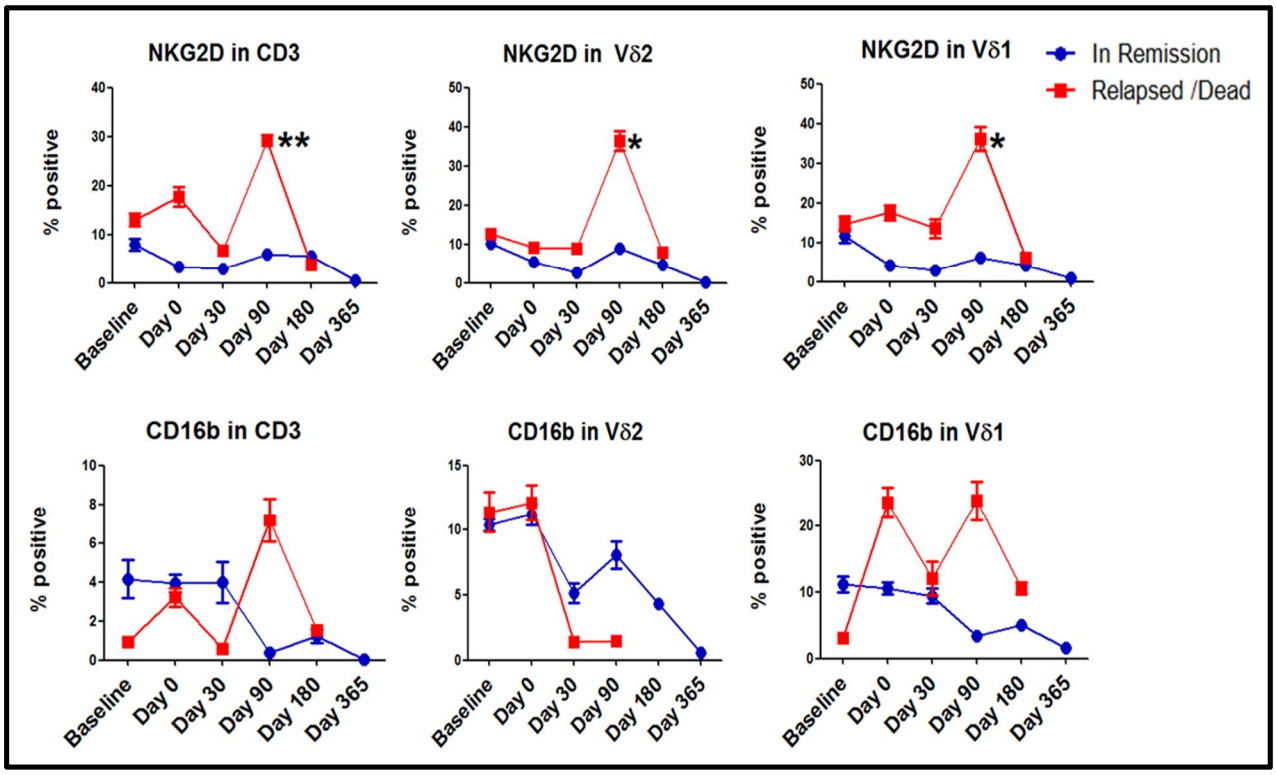


Figure 7.7: Expression of NK cell markers pre and post BMT in remission and relapsed/dead patients. Blue line represents patients in remission and red line represents relapsed or dead patients. X axis represents number of days post BMT. Data is represented as mean±SE *: $p<0.05$, **:$p<0.005$

f) Perforin expression

Perforin is a pore forming cytolytic protein found in the granules of Cytotoxic T lymphocytes (CTLs). As shown in figure 7.8, it was observed that perforin expression

is high in CD3, Vδ1 and Vδ2 T cells on day 90 post BMT in patients who relapsed or died as compared to patients who achieved remission.

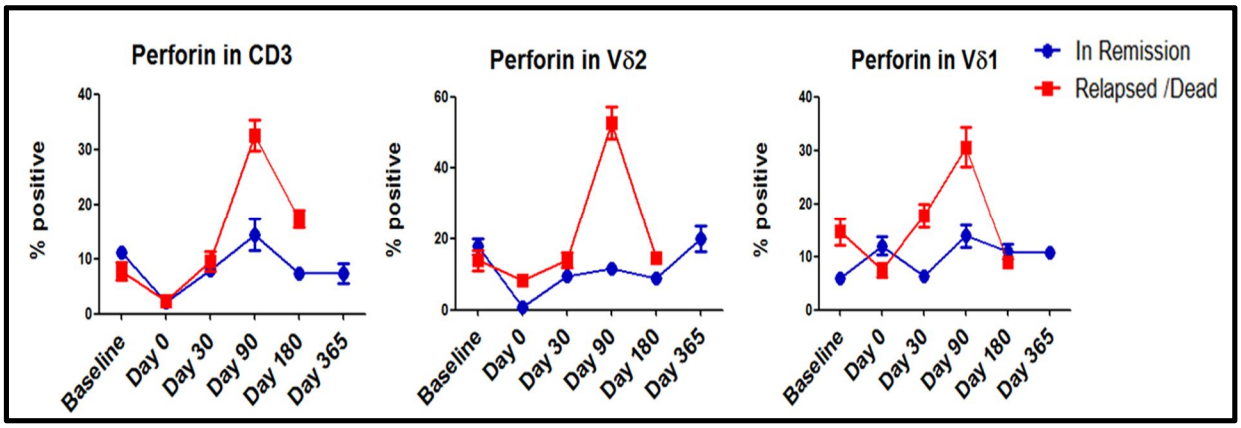


Figure 7.8: Perforin expression in remission and relapsed/dead patients pre and post BMT. Blue line represents patients in remission and red line represents relapsed or dead patients. X axis represents number of days post BMT. Data is represented as mean±SE *: $p<0.05$

7.4 Correlation of clinical parameters with recipient's overall survival and relapse free survival post BMT

Various clinical parameters such as WBC count, CD34 count, platelet and WBC engraftment day, CMV reactivation, chronic and acute GvHD, recipient and donor's age were correlated with OS and RFS of patients post BMT. Two clinical parameters, day of WBC engraftment and chronic GvHD showed significant correlation with OS and RFS in BMT recipients. Recipients with incidence of chronic GvHD, have better OS ($p=0.08$) and significantly higher RFS ($p=0.04$) as compared to patients with no cGvHD (Figure 7.9 A and C). If recipients show earlier WBC engraftment then they show significantly better OS ($p=0.042$) and RFS ($p=0.05$) as compared to patients with late WBC engraftment (Figure 7.9 B and D).

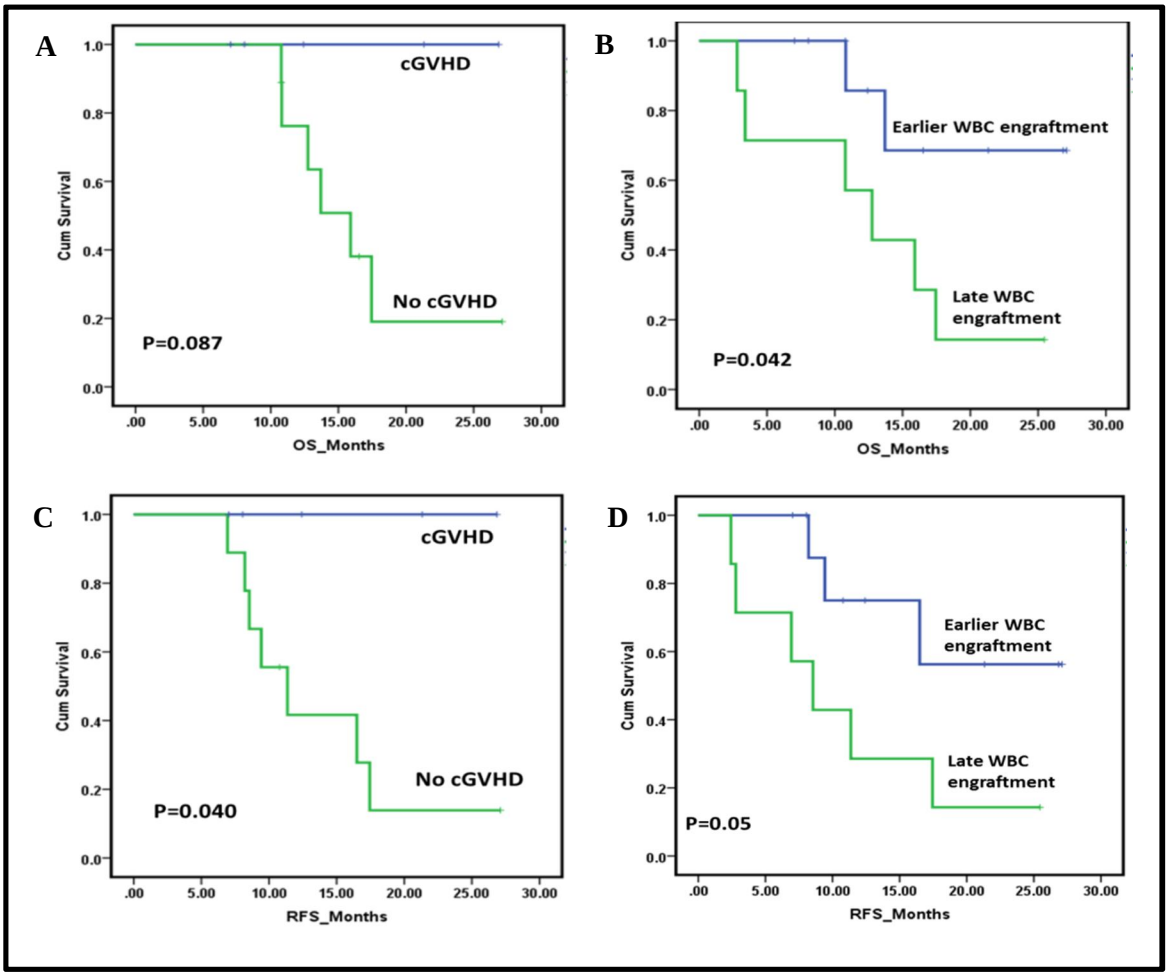


Figure 7.9 Effect of clinical parameters on Overall Survival and Relapse Free Survival. Cumulative survival is calculated using Kaplan Maier survival analysis. Panels A and B indicate OS in months and panels C and D indicate RFS in months. P value in each box indicates statistical significance

7.5 Correlation of recipient's immunophenotype with clinical outcome post BMT

In a retrospective analysis, recipient's immune cell composition at different time points was compared with clinical outcomes such as acute or chronic GvHD, treatment related mortality, WBC engraftment, CMV reactivation or relapse. Patients who exhibited acute GVHD, show significantly high Vδ2/NKG2D (p=0.03) on Day 180, post BMT. Patients with low Vδ2/NKG2D on Day 30 (p=0.028); high CD3 CM (p=0.048), and high NKT (p=0.048) on Day 90; and low CD3/CD16b on Day 180 (0.048) show presence of chronic GVHD. Patients who showed earlier WBC engraftment (< 15 days) show distinct immune reconstitution than

the patients with late engraftment. Patients with early WBC engraftment show high NK (p=0.021) and high CD19 (p=0.021) on day 30; low CD3/NKG2D (p=0.008), low Vδ2/NKG2D (p=0.008), and low Vδ1/NKG2D (p=0.008) on Day 90, post BMT as compared to those with late WBC engraftment. Patients with CMV reactivation post BMT, have low Vδ2 TemRA on Day 90 (0.048) as compared to those without CMV reactivation (Table 7.2).

Table 7.2: Correlation of recipient's immunophenotype with clinical outcome

Acute GVHD	Chronic GVHD	Early WBC engraftment	CMV reactivation
High Vδ2/NKG2D on Day 180 (0.033)*	Low Vδ2/NKG2D on Day 30 (0.028)	High NK on Day 30 (0.021)	Low NKT on Day 30 (0.070)
	High CD3 CM on Day 90 (0.048)	High CD19 on Day 30 (0.021)	Low CD3/CD25 on Day 30 (0.070)
	High NKT on Day 90 (0.048)	Low CD3/NKG2D on Day 90 (0.008)	Low Vδ1 CM on Day 30 (0.070)
	Low CD3/CD16b on Day 180 (0.048)	Low Vδ2/NKG2D on Day 90 (0.008)	Low Vδ2/CD69 on Day 30 (0.070)
		Low Vδ1/NKG2D on Day 90 (0.008)	Low Vδ2 TemRA on Day 90 (0.048)
* : P value			

Overall recipient's immunophenotpe till day 90 post BMT can be used as a predictor of cGVHD.

Patients who showed earlier WBC engraftment (within 15 days) show distinct immune reconstitution than the patients with late engraftment.

7.6 Correlation of donor's and recipient's immunophenotype.

Peripheral blood of donors of patients receiving allogeneic BMT was collected before BMT. Immunophenotyping was carried out for donors at baseline. Difference in immunophenotype of donors was studied and these were categorised in two groups, 1.Donors whose recipients

achieved remission and 2. Donors whose recipients relapsed/died post BMT. Immune cell markers which were expressed at significantly different levels between remission and relapsed/dead patients (as described in section 7.3) were studied for differences in expression of these markers in their respective donors. Two significant immunophenotypes (NKT cells and Vδ1 TemRA cells) are explained in this section.

A. NKT cells

As described in figure 7.3, NKT cells were expressed significantly higher in patients achieving remission. Further their respective donors were compared with each other. It was observed that recipients who achieved remission show significantly high expression of NKT cells as compared to their respective donors as well as donors of relapsed/dead patients on day 30 post BMT (Figure 7.10). Donors of patients who achieved remission show significantly high NKT cells compared to donors of patients who relapsed/died.

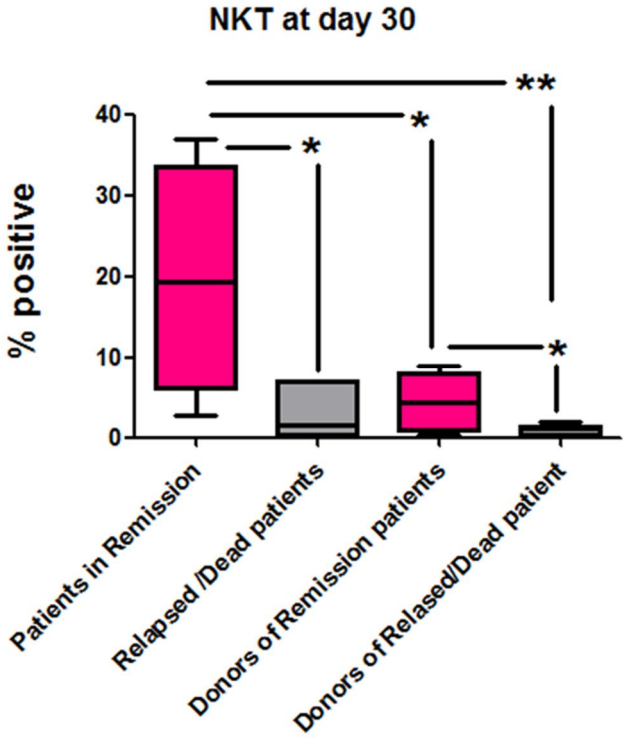


Figure 7.10 Comparison of NKT cells in donors and recipients. % positive expression of NKT cells on day 30 post BMT was studied. Pink bars represent patients in remission and their respective donors, grey bars represent relapsed/dead patients and their respective donors. Box whisker plots showing minimum to maximum range with a mean. *: $p<0.05$, **:$p<0.005$

B. Memory markers

As described in figure 7.6, patients in remission have significantly high Vδ1 TemRA cells as compared to relapsed/dead patients. Donors of patients who achieved remission also have significantly high Vδ1 TemRA cells as compared to donors of patients who relapsed/died. If donors had high Vδ1 TemRA cells at baseline, then the respective recipients have significantly more chance of achieving remission post BMT (Figure 7.11).

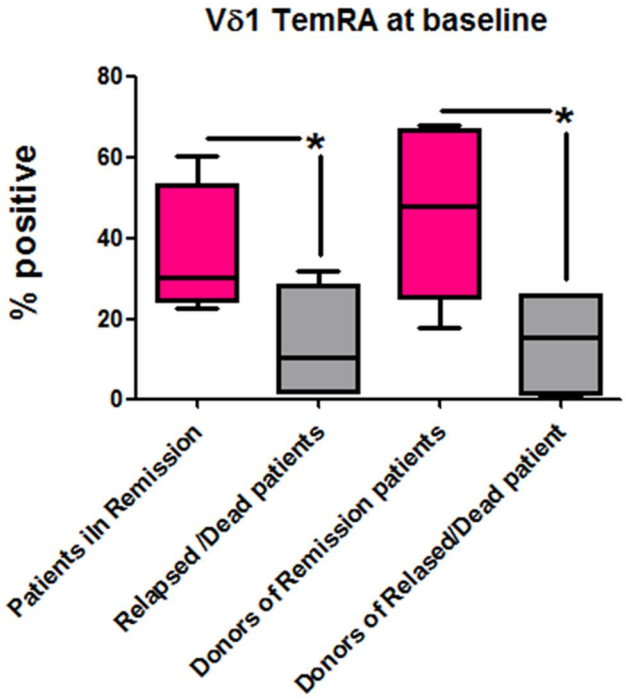


Figure 7.11 Comparison of memory markers in donors and recipients. % positive expression of TemRA+ Vδ1 T cells at baseline was studied. Pink bars represent patients in remission and their respective donors, grey bars represent relapsed/dead patients and their respective donors. Box whisker plots showing minimum to maximum range with a mean. *: $p<0.05$

7.7 Correlation of donor's immunophenotype with clinical outcome post BMT

Donor's immune cell composition plays important role in building the immune system of recipient. As recipient's immune system is ablated, donor's immune cells repopulate and decide the clinical outcome of recipient such as incidence of acute or chronic GvHD, relapse, and treatment related mortality, or CMV reactivation.

a) Correlation of donor's immunophenotype with GvHD and relapse

If donors had high CD3 EM and low Vδ1/NKG2D, recipients develop acute GVHD post BMT (p=0.028). Donors with high CD3 (p=0.032), high Vδ1 and Vδ2 Perforin (p=0.035) have significant correlation with treatment related mortality. Recipients are more likely to exhibit TRM, if they receive the graft with these immune cells. Recipients show relapse, if donors had low NKT cells (p=0.034), low NK (p=0.045), and low CD3 Perforin (p=0.023). Hence donor's immune cell composition affects the clinical outcome of recipients and can be used as predictors of outcome in recipients (Table 7.3).

Table 7.3: Correlation of Donor's immunophenotype with clinical outcome

Acute GVHD	TRM	Relapse
High CD3 EM (0.028)*	**High CD3 (0.032)**	**Low NKT (0.034)**
Low Vδ1/NKG2D (0.028)	**High Vδ1 Perforin (0.035)**	**Low NK (0.045)**
High CD3 Perforin (0.072)	**High Vδ2 Perforin (0.035)**	**Low CD3 Perforin (0.023)**
Low CD3 Naïve (0.072)		Low CD3 (0.089)
Low V**δ**2 CM (0.062)		Low V**δ**2 perforin (0.089)
High V**δ**2 EM (0.062)		
* : P value		

b) Effect of donor's immunophenotype on cumulative incidence of CMV post BMT

Human cytomegalovirus (CMV) is a widely prevalent herpes virus that, after primary infection, persists lifelong in the human host. Although infections are asymptomatic in most immunocompetent individuals, reactivation of the virus in immunocompromised patients after allogeneic stem cell transplantation (allo-SCT) can lead to life-threatening complications including colitis and pneumonia [212]. It was observed that donor's immune cell composition correlates with cumulative incidence of CMV in recipients, post

BMT. Donors with high γδ T cells, show significantly less CMV reactivation (p=0.022) in recipients (Figure 7.12 A). Hence γδ T cells are playing important role to prevent CMV reactivation in recipients. Recipients show significantly less CMV reactivation, if donors had high CD19 (p=0.05), CD4 T cells (p=0.02) (Figure 7.12 B, C).

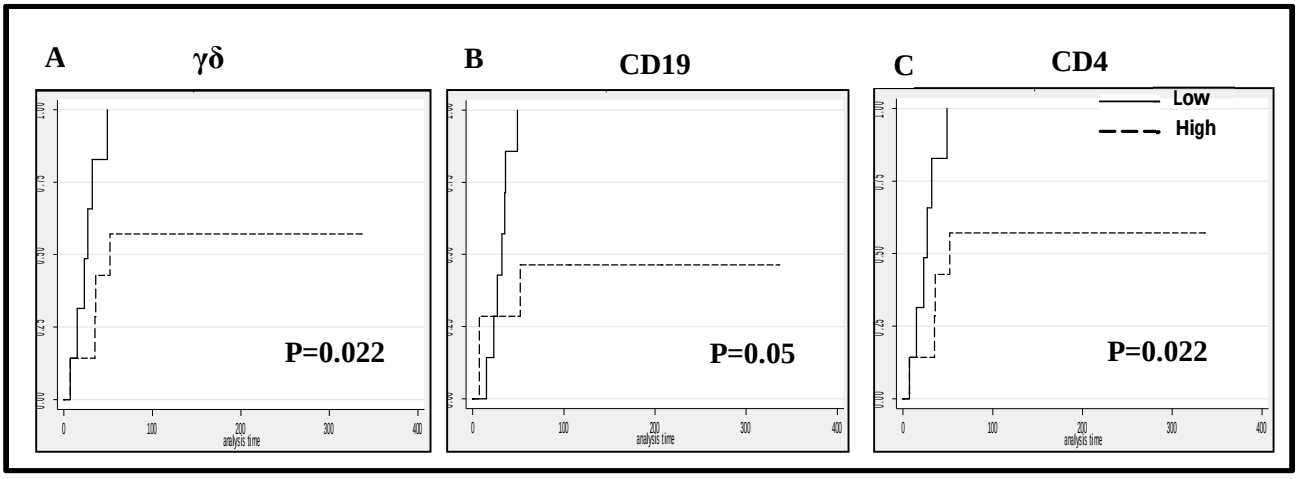


Figure 7.12: Correlation of donor T and B cell composition on cumulative incidence of CMV. Cumulative incidence of CMV in recipients, post BMT was calculated using Kaplan Maier failure analysis based on the expression of different immune cell compositions in donors. Cumulative incidence of CMV is plotted against number of days post BMT. P value in each box indicates statistical significance.

Recipients show significantly less CMV reactivation, if donors had high naïve Vδ2 (p=0.04, figure 7.13 A), Vδ2 TemRA (p=0.02, figure 7.13 C); and low CD3 EM (p=0.01, figure 7.13 D) and Vδ1 EM (p=0.001, figure 7.13 B) cells.

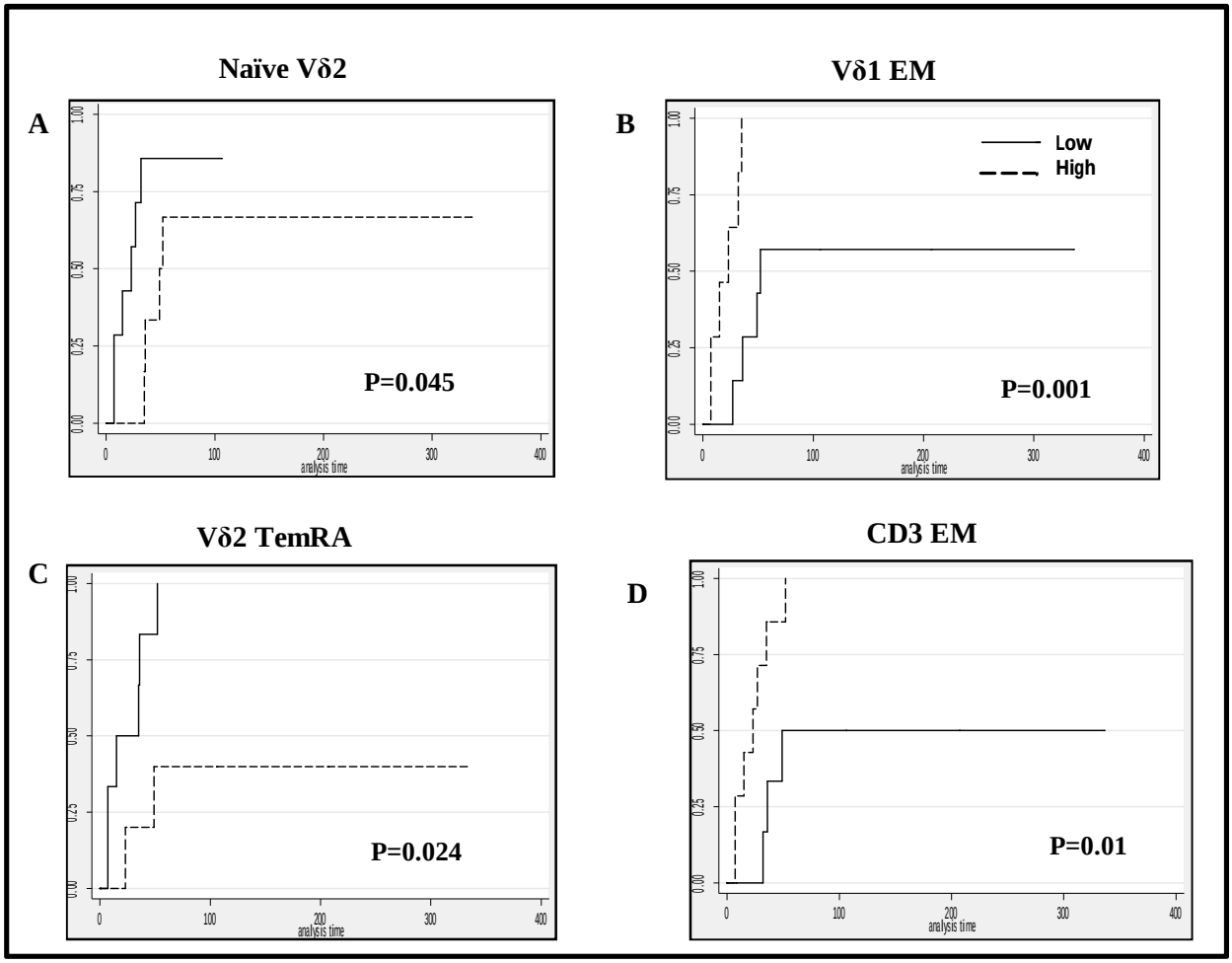


Figure 7.13: Correlation of memory markers with cumulative incidence of CMV. Cumulative incidence of CMV in recipients, post BMT was calculated using Kaplan Maier failure analysis based on memory markers in donors. Cumulative incidence of CMV is plotted against number of days post BMT. P value in each box indicates statistical significance.

Activation status of donor's immune cells correlates with cumulative incidence of CMV reactivation. Recipients show less CMV reactivation if donors have high expression of CD3/CD25 (p=0.013, figure 7.14 A) and Vδ2/CD25 (p=0.04, figure 7.14 B)

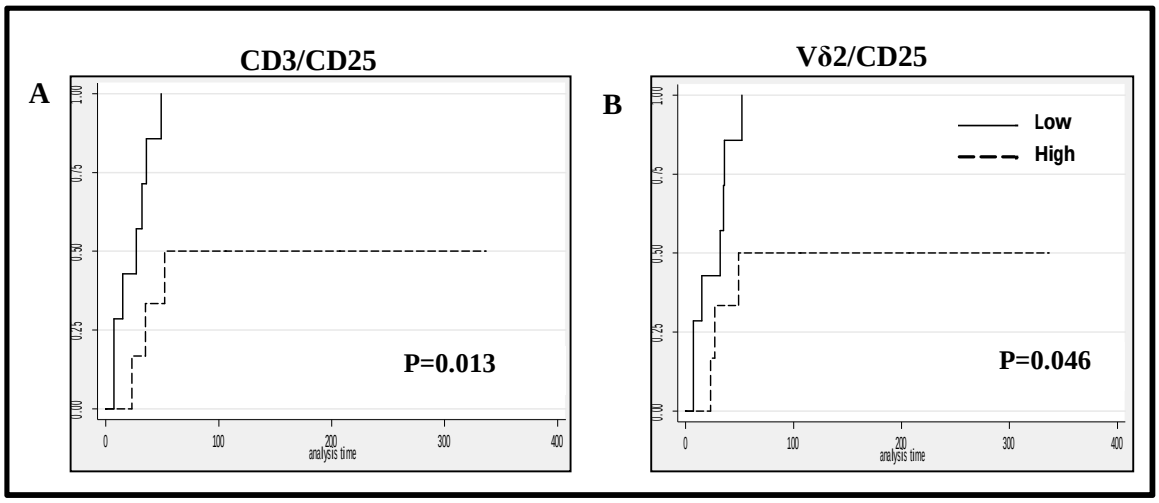


Figure 7.14: Correlation of activation markers with cumulative incidence of CMV. Cumulative incidence of CMV in recipients, post BMT was calculated using Kaplan Maier failure analysis based on activation markers in donors. Cumulative incidence of CMV is plotted against number of days post BMT. P value in each box indicates statistical significance.

These donor's immune cell markers can be used to predict the CMV reactivation in recipients post BMT.

In conclusion, patients with earlier WBC engraftment and cGVHD, show better OS and RFS. Donor's immune cell composition affects the clinical outcome of recipients. Recipients show significantly less CMV reactivation, if donors had high γδ T cells, CD19, CD4, Naïve Vδ2, Vδ2 TemRA, CD3/CD25, and Vδ2/CD25; and low CD3 EM, and Vδ1 EM. Hence γδ T cells play important role in recipient's outcome post BMT.

CHAPTER 8

Discussion

Cancer is a disease caused by the uncontrolled growth of a single cell. This growth is unleashed by the mutations in the genes that incite unlimited cell growth. In a normal cell powerful genetic circuits regulate cell division and cell death. In a cancer cell, these circuits have been broken, unleashing a cell that cannot stop growing. Leukemia is cancer of white blood cells, cancer in one of its most explosive, violent incarnations.

Immune dysfunction in the cancer-bearing host can promote tumor cell variants that are able to resist or suppress antitumor immune responses, leading to tumor progression [213]. The leukemias represent ideal models to assess the impact of cancer on the host immune system as the disease is widely disseminated so that immune cells in the peripheral blood (PB) are in close proximity to the tumor cells and can be readily sampled [214].

T-ALL is phenotypically and clinically heterogenous disease with a high frequency of treatment failure. Efforts have been focused on identifying biological predictors of disease prognosis and response to treatment as well as molecular targets for innovative therapeutic approaches. Our earlier studies have identified clonal TCR γ and δ junctional gene rearrangement status of T-ALL patients at diagnosis as a prognostic marker and predictor of response to chemotherapy. Our long term follow up study showed that TCRγδ+ T-ALL patients have survival benefit, over TCRαβ+ T-ALL patient cohort [18-20].

The present study was aimed at investigating the immune scenario in T-ALL patients at diagnosis and remission and to compare these profiles with HI. The study was further extended to understand the genotypic differences in TCR γδ clonal and TCR αβ clonal leukemic blasts and understand how they influence the immune scenario in T-ALL patients. The study also focused on understanding the role of γδ T cells in allogeneic bone marrow transplantation.

To unveil the reason behind the survival benefit to TCRγδ+ T-ALL; in the present study we performed gene expression profiling as well as analysis of mutation and copy

number variation in selected genes of these two T-ALL subsets. We also performed functional studies to analyse differences in immune scenario in these subsets of T-ALL. Our results have shown distinct genomic and functional signatures of TCRγδ+T-ALL and TCRαβ+T-ALL subgroups when compared with each other and also with normal lymphocyte profile [209].

Immunophenotype of T-ALL patients is reported with respect to surface expression of TCR complex [215], but total immune scenario in T-ALL patients according to their TCR clonotype (TCRαβ and TCR γδ) is not yet reported. We studied immune composition of T-ALL patients at different stages of disease viz., at diagnosis and at remission and compared it with healthy individuals. We also analysed the difference in immunophenotype of TCRγδ+T-ALL and TCRαβ+T-ALL subgroups.

T-cell acute lymphoblastic leukemia (T-ALL) results from malignant transformation of immature cells of the T-cell lineage. Immature thymocytes fail to show presence of membrane receptors of CD3, CD4, and CD8 in most cases. In present study it was observed that T-ALL patients at diagnosis showed significant low percentage of CD3+, T helper, T cytotoxic, αβ T, γδ T, NK and NKT cells compared to patients who achieved remission and healthy individuals. This indicates deregulated immune system in patients at diagnosis. γδ T cell subsets Vδ1 and Vδ2 which play important role in recognition and killing of leukemic blasts, were significantly high in patients at remission compared to patients at diagnosis. Early and late activation markers (CD69 and CD25 respectively) were also elevated on CD3, Vδ1 and Vδ2 T cells in patients at remission indicating presence of functionally activated cells.

Upon antigenic stimulation and activation of naive T cells, asymmetric cell division differentiates effector and memory T-cell fates. Memory T cells can be divided into at least 3 distinct subsets. The $CD45RA^{-}CD27^{+}$ “central” memory (CM) population migrates

preferentially through lymphoid tissue and is long-lived, whereas the $CD45RA^{-}CD27^{-}$ "effector" memory (EM) population migrates preferentially in non-lymphoid tissues and is short-lived. A $CD45RA^{+}CD27^{-}$ "late effector" memory (TemRA) population has been identified in humans and is closely related to the EM population [216]. EM cells show the highest IFN-γ secretion, while TemRA cells are characterized by a strong cytotoxic activity. In contrast, naive cells display very low, if any, functional activity [217-219], but their clonal plasticity remains uncertain. The central memory subset homing to the lymph node and lacking effector functions may represent an antigen-primed Vδ2 population trafficking to the lymph nodes and upon encounter with antigen generates a new wave of effector cells. On the other hand, EM cells represent a readily available pool of antigen-primed Vδ2 T cells which enter the peripheral tissues, where they can eventually further differentiate to TemRA cells, and by their ability to produce cytokines and exert cytotoxicity can contribute to the containment of invading microbial pathogens [217].

Functional exhaustion has been associated with a differentiation shift, which is well in line with the accumulation of EM/TEMRA Vγ9Vδ2 T cells in CLL patients [220]. Long-lasting tumor induced chronic activation leads to the undesired accumulation of these cells unable to exert effective antitumor activity [220]. AML patients at diagnosis had high proportion of EM cells and normal naive and CM Vγ9Vδ2 T cell proportions (CD27+) that were restored in a leukemia-free environment, that is, in patients undergoing remission after chemotherapy. These results suggest that recognition of leukemic blasts could induce the differentiation of Vγ9Vδ2 T cells into effector cells [30]. In accordance with these reports, we observed significantly low number of naïve and CM cells and high EM cells in patients at diagnosis as compared to patients at remission as well as healthy individuals. Patients at remission showed significant high numbers of terminally differentiated (TemRA) cells compared to patients at diagnosis. This indicates low proliferative and cytotoxic potential of

immune cells in patients at diagnosis, confirming immune dysfunction at diagnosis and its restoration at remission.

In the present study we found a significant increase in CD16b and NKG2D receptors on total CD3 and γδ T-cell subsets in patients at remission. NKG2D is a C-type lectin-like type receptor and belongs to the NK group 2 (NKG2) of receptors as member D. It has also been classified as killer cell lectin-like receptor of the subfamily K, member 1 (KLRK1) [221]. NKG2D is a homodimer and recognizes a number of stress induced MHC class I-like ligands [222, 223]. There is no inhibitory counterpart known for NKG2D and NKG2D is capable of overriding signals provided by inhibitory receptors on NK cells engaging MHC class Ia and Ib molecules. Thus, NKG2D plays a role as a molecular sensor detecting "induced self" on cells in danger, which is mostly triggered by viral infections and by factors causing DNA damage and tumor transformation [222, 224, 225]. Like NK cells, human γδ T cells also express the CD16b (FcγRIII) receptor that binds to the Fc portion of immunoglobulin G (IgG). CD16b expression on Vγ9Vδ2 T cells can be up-regulated following stimulation with phosphoantigens [226]. Its engagement leads to antibody dependent cellular cytotoxicity (ADCC), a process that can result in lysis of tumor cells bound by specific antibodies. Indeed, several in vitro studies have clearly shown that γδ T cells are activated through CD16b and mediate ADCC of tumor cells in the presence of therapeutic antitumor monoclonal antibodies [227]. Given their marked ability to engage and to lyse tumor cell targets, their high content of perforin, and their ability to secrete TNF-α and IFN-γ upon CD16-mediated (but not phosphoantigen-mediated) activation, the terminally differentiated Vδ2 TemRA cells represent a distinct and critical pool of cytotoxic effectors within the γδ T-cell population [219]. In the present study we observed that, as the immune effector cells are increased significantly, expression of perforin (Perforin is a pore forming

cytolytic protein found in the granules of Cytotoxic T lymphocytes and NK cells) was also increased in T-ALL patients in remission.

T-ALL patients in remission showed comparable levels of Vδ1 and Vδ2 T cells as that of healthy individuals. Although the numbers of immune cells are significantly increasing in remission state compared to patients at diagnosis, immune system of patients in remission do not match with that of healthy individuals. This may be due to presence of undetectable residual blast cells or immunosuppressive effect of chemotherapy. Overall γδ T cells are playing an important role in remission patients, as the number of activated γδ T cells and the expression of perforin molecules in γδ T cells is increased compared to patients at diagnosis.

We observed significant differences in immunophenotypes of TCRγδ+T-ALL and TCRαβ+T-ALL subgroups. TCRγδ+T-ALL patients showed significantly high expression of Vδ2 T cells, NKT cells, γδ T cells as compared to TCRαβ+T-ALL subgroup. The immune cells in TCRγδ+T-ALL subgroup showed significantly increased levels of late activation marker on immune cells (CD25+) and also high expression of NKG2D receptor. We observed that Vδ2 and Vδ1 T cells express higer level of perforin molecules in TCRγδ+T-ALL subgroup. It was reported that the $CD45RA^{-}/CD45RO^{+}$ phenotype is the most common in γδ T-ALL in both children and adults, whereas the $CD45RA^{+}/CD45RO^{-}$ phenotype predominated in αβ T-ALL, when TCR complex is expressed on surface [215]. Similarly we observed significantly higher expression of CM ($CD27^{+}CD45RA^{-}$) and TemRA ($CD27^{-}CD45RA^{+}$) cells in TCRγδ+T-ALL subgroup as compared to TCRαβ+T-ALL. Overall the survival advantage to TCRγδ+T-ALL subgroup can be at attributed to the underlying difference in immune composition generated in the host between the two subgroups of T-ALL.

Further we also studied antigens expressed on leukemic blast cells from T-ALL patients. Mainly the known ligands of Vγ9Vδ2 T cells (HSP60, HSP70, MICA, MICB, ULBP1, and CD166) were studied before and after aminobisphosphonate (Zoledronate) treatment. Aminobisphosphonates inhibit the farnesyl pyrophosphate synthase (FPPS) enzyme in the mammalian mevalonate pathway, allowing accumulation of endogenous IPP in tumor cells and resulting in activation of Vγ9Vδ2 T cells [128]. Vγ9Vδ2 T cells have the prerogative to be activated not only via TCR-dependent stimuli but also via TCR-independent interactions of stress pathway receptors, such as NKR, with the corresponding ligands on tumor cells. NKG2D is an activating NKR recognizing ligand such as MICA/B and ULBP, which are expressed on the cell surface of several hematologic malignancies including CLL [228, 229].

ULBP molecules have been involved in the recognition by Vγ9Vδ2 T cells of leukemia and lymphoma [163] and also of solid tumors, such as ovarian and colon carcinomas [230, 231]. For instance, ULBP1 expression level determines lymphoma susceptibility to γδ T cell-mediated cytolysis upon NKG2D binding [163]. ULBP and MICA interact with NKG2D or TCR on Vδ1 γδ T cells and induce their activation. ULBP1 is a ligand for the NKG2D receptor expressed on all cytotoxic lymphocyte lineages, including 100% of Vγ9Vδ2 T cells, which has been clearly implicated in anti-tumor responses [223, 232]. Recognition of MICA, MICB, or ULBP expressed on cancer cells by human Vγ1δ1 (Vδ1) T lymphocytes can trigger or increase their cytolytic activity against tumor cells that express NKG2D ligands [145, 228]. Indeed, ULBP and MICA interact with NKG2D or TCR on Vδ1 γδ T cells and induce their activation. However, MICA binds in mutually exclusive manner to NKG2D and TCR, suggesting that the two receptors might be sequentially engaged following recognition of target tumor cells [231].

In the present study, we found significant increase in the expression of CD166 on leukemic blasts after Zoledronate treatment. CD166 (also called activated leukocyte-cell adhesion molecule) broadly expressed on the human tumor cell lines is a candidate molecule involved in γδ T cell activation and suggest that the engagement of CD6 on γδ T cells with CD166 may play a significant role in γδ T cell activation by the tumor cells loaded with nonpeptide antigens, either endogenously or extracellularly [233].

To mount an effective immune response, immune cells need to communicate with each other. One way in which this is done is by the formation of immunological synapses between cells [234]. The immunological synapse (IS) is a specialized cell–cell junction between a thymus-derived lymphocyte (T cell) and an antigen-presenting cell (APC) [235]. Immunological synapse impairment is clearly used as an immunomodulating mechanism by tumor cells, and it can be speculated that this can facilitate disease progression in the hostile immune environment of the host [236]. T cell antigen receptor engagement and recognition of antigen induces dramatic morphological changes in T cells, characterized by polarization of the actin cytoskeleton and accumulation of F-actin at the site of contact with the APC [213].

LFA-1 plays a key role in the structure of the IS and is also likely to play a key role in signalling [157]. The dynamin–actin complex is at the heart of many important motile cell processes. This complex is likely to possess contractile properties while providing multiple sites to recruit or bind to a variety of cytoskeletal and adaptor proteins to the membrane [237]. Dynamin-2 mediates F-actin polymerization at the immunological synapse and subsequent regulation of signals controlling TCR-mediated T cell activation [238]. Recently mutations in dynamin-2 were detected in T-ALL patients that were associated with high-risk leukemia [88]. These defects in dynamin-2 molecule may affect immune synapse formation in T-ALL patients. In our study we showed increased F-actin polarisation at IS when

leukemic blasts were treated with Zoledronate. Interestingly significant difference in MFI of F-actin was observed at IS between γδ T cells and Zoledronate treated TCR γδ clonal leukemic blasts as compared to TCR αβ clonal blasts. Similarly LFA-1 expression was also high at IS between Zoledronate treated TCR γδ clonal leukemic blasts and γδ T cells.

CD6 is a member of scavenger receptor cysteine-rich protein superfamily expressed on T cells and some B cells [239] and was suggested to be involved in the thymocyte adhesion to thymic epithelial cells as well as T cell activation. γδ T cells express CD6, a receptor of CD166. CD166 expression on tumor cells provides a potent costimulatory signal for TCR-dependent Vγ9Vδ2 T-cell activation by recruiting CD6 at the centre of the immune synapse colocalizing with γδ TCR/CD3 [233]. Besides TCR and NKR signaling, Vγ9Vδ2 T-cell activation is also regulated by the interactions of cell surface receptors with adhesion molecules such as ICAM-1 and CD166 [233, 240]. Kato et al have shown that expression of CD166 on tumor cells increases after aminobisphosphonate treatment [233]. Similarly we observed increased expression of CD166 on TCR γδ clonal leukemic blasts after Zoledronate treatment as compared to TCR αβ clonal leukemic blasts. It can be argued that the lower CD166 expression on TCR αβ clonal blasts induced by Zoledronate may be resulting in reduced activation of Vγ9Vδ2 T cells. Similarly it has been reported by Coscia et al that low responder CLL patients expressed lower CD166 on tumor cells which was linked to defects in mevalonate pathway in these patients [220].

As soon as the conjugates are formed, TCR-mediated activation triggers membrane stripping and granule exocytosis, both of which persist beyond target cell death. In γδ T cell synapses, the release of perforin is triggered very early by TCR activation, although the lytic IS is maintained for much longer [241]. In the live cell time lapse imaging, we observed higher expression of perforin molecules at IS between γδ T cells and Zoledronate treated TCR γδ clonal leukemic blasts as compared to TCRαβ clonal blasts.

In our gene expression analysis, we observed significant differences in expression of genes involved in immune synapse formation (CD6, CDC2, ICAM-1 etc) between TCRαβ+ T-ALL and TCRγδ+ T-ALL patients. This indicates inherent ability of TCRγδ clonal leukemic blasts to express higher levels of immune synapse related molecules on their surface. Leukemic blasts from TCRγδ+T-ALL patients after Zoledronate treatment showed higher F-actin polarisation, CD166 and LFA-1 expression at IS as compared to blasts from TCRαβ+T-ALL patients. This suggest better IS formation and more susceptibility of TCR γδ clonal leukemic blasts to lysis, when co-cultured with γδ T cells. As observed in co-culture experiments, TCR γδ clonal leukemic blasts expand more numbers of Vδ2 T cells when co-cultured with healthy PBMCs, indicating that these blasts express γδ T cell specific antigens on the surface.

Comparative genomic studies in TCRγδ versus TCRαβ clonal T-ALL patients were carried out using cDNA microarray, real time PCR and ddPCR. We observed district gene expression, Copy number variation (CNV) and mutation patterns in TCR γδ+T-ALL as compared to TCR αβ+T-ALL patients. Various genes involved in important pathways such as apoptosis, leukemia formation, survival, cytokine signalling etc were found to be significantly differentially regulated between these two subgroups of T-ALL patients. In our study pro-survival *NFKB1* gene was found to be significantly upregulated in TCRαβ+ T-ALL patients compared to TCRγδ+T-ALL subgroup. Other reports have demonstrated *NFKB1* activation to counteract apoptosis induced by TNF, Fas ligation, cytotoxic drugs, and other stimuli and block activation of caspase 8, a proteolytic enzyme involved in the apoptotic cascade [242]. *NF-KB* activation is important for the induction of T-cell leukemia, as suppression of the *NF-KB* pathway substantially reduces the severity of the disease [243].

Our results also show significant up regulation of survival related genes *BAX*, *HRK* and *FAS* in the TCRγδ+T-ALL patient subgroup. The susceptibility of cells to apoptosis

depends on the relative expression of intracellular molecules that enhance apoptosis (pro-apoptotic), with BAX being the major representative molecule [244, 245]. In accordance with our data, Irvine et al also observed that AML patients with high *BAX* expression at diagnosis exhibited better event-free survival and overall survival [246]. Further Sanz et al demonstrated that *HRK* acts as a trigger for apoptosis and provokes cell death in hematopoietic progenitors even in the presence of survival factors [247]. While, in some paediatric ALL studies increased levels of *FAS* were shown to be associated with longer survival and complete remission [248].

Mutation and CNV analysis corroborated our earlier finding that TCRγδ+T-ALL is a distinct subgroup of T-ALL than TCRαβ+T-ALL. To the best of our knowledge, such an extensive analysis using clonality status, mutation/CNV and survival has not been reported earlier. Activating mutations in *NOTCH1* have been shown to occur in more than 50% of T-ALL cases [67]. We also observed similar results in our T-ALL cases when compared to normal lymphocytes. There are several conflicting reports on *NOTCH1* mutation and survival [249-251]. In the present study, association of survival with *NOTCH1* mutation was not statistically significant.

IKZF3 mutations have not been reported in T-ALL, but are reported in CML, B-ALL and B-CLL [252]. In our study, we found mutation in *IKZF3* gene at very low concentration in T-ALL patients. The importance of detecting *IKZF3* mutations in T-ALL lies in the fact that multiple therapeutic targets which are being investigated in patients with Ikaros mutation [253-255] could be of potential therapeutic benefit in patients with T-ALL.

Various previous reports have demonstrated that *BRAF* mutations are rare in hematologic malignancies including leukemias and lymphomas [256, 257]. As we used highly precise and sensitive technique of ddPCR; we could detect very low level mutations in *BRAF* gene. Gustafsson et al [258] reported no association between mutations in *BRAF* and

DFS or any other clinical parameter. Similarly, we observed no significant difference in DFS based on BRAF mutation.

In our study, TCRγδ+T-ALL patients with wild type/heterozygous deletion of *CDKN2A/B* possess significantly better DFS over TCRαβ+T-ALL patients. While in both the subgroups, homozygous deletion of *CDKN2A/B* showed inferior survival as compared to wild type/heterozygous copies, underscoring the importance of these tumor suppressor genes in Indian T-ALL cohort. The prognostic significance of CDKN2A deletions in ALL appears debatable. Vlierberghe et al [249] have shown that homozygous deletion of CDKN2A/CDKN2B was associated with favorable outcome in T-ALL, whereas such correlation was not reported by others [259, 260]. In accordance with our observations, deletion/methylation of *CDKN2A/B* was found to correlate with poor survival in Follicular Lymphoma patients [261], in Adult BCR-ABL1–Positive ALL [262] and in adult B-ALL [263]. Present study has reported for the first time, TCR clonality status in combination with CDKN2A/CDKN2B CNV to be associated with DFS in Indian T-ALL patients.

Allogeneic hematopoietic stem cell transplantation (HSCT) has improved disease-free survival in patients with refractory acute leukemia, or those at a high risk of relapse. Over the past several years, it became apparent that the donor graft, in addition to its role in replacement of the ablated hematopoietic system, elicits an immunotherapeutic benefit (graft-versus-leukemia effect (GvL). The role of T cells as mediators of GvL was recognized when T cell depletion of hematopoietic grafts was found to be associated with an increased risk of relapsed disease, especially in patients with chronic myeloid leukemia [264-267]. We studied the role of γδ T cells in T-ALL and B-ALL patients receiving allogeneic BMT and correlated it with the disease outcome. γδ T cells may provide an ideal source of T cell immunotherapy for leukemia and are advantageous in allogeneic BMT, as γδ T cells can: (i) detect and sense any type of stress through a MHC-independent mechanism, (ii) produce

huge quantities of pro-inflammatory cytokines, and (iii) exert potent cytotoxic activity against a broad panel of tumors [127].

We observed that γδ T cells play important role in prevention of CMV reactivation in recipients post BMT. Recipients in our study showed significantly less CMV reactivation ($p=0.022$) when their respective donors had high γδ T cell counts. Specifically donors with high CD25+, naïve and TemRA Vδ2 T cells show significantly less CMV reactivation in recipients. Previously the role of Vδ1 T cells is reported in CMV reactivation. Vδ1 T cells reside within epithelial tissues, especially at sites of cytomegalovirus (CMV) replication, and exert potent cytotoxic effects against acute lymphoblastic leukemia (ALL) or AML cells [27], chronic lymphocytic leukemia cells [29, 268, 269] and primary multiple myeloma cells [50]. Dechanet-Merville and collaborators found that a human δ2 negative T cell subset recognizes both CMV-infected and transformed cells through the interaction between the endothelial protein C receptor (EPCR) and the TCR [270]. EPCR is over-expressed in CMV-infected endothelial cells and transformed cells and it is conceivable that it might act as a determinant of stress surveillance during epithelial cell transformation to communicate a state of "dysregulated self" to γδ T cells. After primary infection, this beta herpesvirus is not eradicated but establishes life-long infection in its host. CMV is dispersed and becomes dormant in multiple end organs; a state also referred to as "latency," and can later be reactivated by a number of different stimuli, including immunosuppression and inflammation [271]. Donor and/or recipient CMV seropositivity is still associated with an adverse prognosis in de novo acute leukemia patients after allo-SCT despite the implementation of sophisticated strategies for prophylaxis, monitoring, and (pre-emptive) treatment of CMV [272].

Alloantigens are recognized more effectively by the naïve cell population than by memory populations, while protection against pathogens is mediated more effectively by

memory populations than by the naïve cell population [216]. As compared with naïve cells, EM cells have a reduced ability to cause GVHD in mice. There are contradictory reports on CM cells, with some studies showing that they cause no GVHD and others showing that they cause less severe GVHD as compared with naïve cells [273]. However in our study we found that higher number of CD3 EM cells in donors causes acute GVHD in recipients. Recipients experience chronic GVHD if there is a higher number of CD3 CM on day 90 post BMT.

We also studied the effect of various clinical parameters on survival post BMT. Recipients with earlier WBC engraftment (<15 days) show significantly better OS and RFS, indicating earlier immune reconstitution in these patients post BMT. Patients, who experienced chronic GVHD, showed significantly better RFS and higher OS. This is in accordance with earlier studies that have shown that the occurrence of chronic GVHD (cGVHD) can improve outcomes in comparison with patients without cGVHD [274, 275]. There are some contradictory earlier studies which reported that low incidence of cGvHD represents a good transplant performance status and better quality of life [276, 277], whereas Jalali et al showed no significant effect of cGVHD on relapse [278]. We observed that clinical parameters such as aGVHD, treatment related mortality (TRM) and relapse are affected by immune cell composition of donors. Recipients experienced aGVHD, when donors have high CD3 EM and low number of Vδ1/NKG2D cells. High expression of lytic protein perforin on Vδ1 and Vδ2 T cells of donors showed TRM in recipients. Recipients experience relapse if donors have low numbers of NK, NKT and CD3/perforin cells, underscoring the importance of these immune cells in relapse free survival. Natural killer (NK) cells are the first lymphocytes to recover after transplantation and are considered powerful effector cells in HSCT [279]. Minculescu et al have shown the independent protective effect of high early NK cell reconstitution on TRM that translates into improved overall survival after T cell–replete HSCT [279]. Patients who are in remission post BMT,

show distinct immunophenotype as compared to patients who relapse or succumb to death. The fate of patient post BMT is in turn decided by donor's immune cell composition. Interestingly recipients in remission and their respective donors, show significantly higher expression of Vδ1 TemRA cells at baseline as compared to those who relapsed/died post BMT and their respective donors.

In conclusion our study has shown for the first time the genotypic, immunophenotypic and functional differences in TCRγδ+T-ALL and TCR αβ+T-ALL subgroups. The study underscores the importance of using TCR γδ clonality as a prognostic marker at diagnosis in Indian T-ALL patients. We have also shown the anti-leukemic role of γδ T cells in T-ALL patients. Patients undergoing allogeneic BMT can be administered with γδ T cell enriched graft so that there are less chances of relapse, GVHD, and CMV reactivation, whereas more graft versus leukemia effects.

Chapter 9

Summary and Conclusion

Indian ALL patients are treated uniformly with high risk chemotherapy protocols. There is great need of risk-based treatment assignment in these patients so that those patients who have a good prognosis can be spared more intensive and toxic treatment. Risk-based treatment assignment requires the availability of prognostic factors that reliably predict outcome.

In our earlier study on Indian T-ALL patients, we identified clonal TCR γ and δ junctional gene rearrangement status of T-ALL as a prognostic marker using a sensitive PCR-coupled Heteroduplex assay. Clinically, survival probability was found to be significantly higher in TCRγδ+ as compared to TCRαβ+T-ALL patients when γ and δ gene rearrangement was considered as prognostic variable. This observation provided an impetus to investigate how TCRγδ+ T-ALL differs biologically from TCRαβ+ T-ALL subgroup.

The present study has identified TCRγδ+T-ALL as a distinct subgroup from TCRαβ+T-ALL using gene expression profiling. TCRγδ+T-ALL subgroup with wild type/heterozygous copies of CDKN2A/B showed significantly ($p=0.017$ and $p=0.045$, respectively) improved DFS as compared to TCRαβ+T-ALL. This study for the first time has also identified CDKN2A/CDKN2B copy number variations combined with TCR clonality as potential prognostic marker in T-ALL patients.

We analysed the immune scenario in T-ALL patients at diagnosis and remission and compared these profiles with HI. We observed significant low numbers of CD3+, T helper, T cytotoxic, αβ T, γδ T, NK and NKT cells in patients at diagnosis compared to HI. This indicates deregulated immune system in patients at diagnosis. γδ T cell subsets Vδ1 and Vδ2 which play important role in recognition and killing of leukemic blasts, were significantly high in patients at remission compared to patients at diagnosis. Early and late activation markers (CD69 and CD25 respectively) were also elevated on CD3, Vδ1 and Vδ2 T cells in patients at remission indicating presence of functionally activated cells. We observed

significantly low number of naïve and CM cells and high EM cells in patients at diagnosis as compared to patients at remission as well as healthy individuals. Although the numbers of immune cells are significantly increasing in remission state compared to patients at diagnosis, immune system of patients in remission do not match with that of healthy individuals. Overall γδ T cells are playing an important role in remission patients, as the number of activated γδ T cells and the expression of perforin molecules in γδ T cells is increased compared to patients at diagnosis.

We further studied the differences in the two subgroups of T-ALL patients at functional level. We observed that TCRγδ+T-ALL patients show differences in immunophenotype as compared to TCRαβ+T-ALL patients. TCRγδ+T-ALL patients show higher expression of γδ T cell subsets, perforin molecules, CM and TemRA cells, NKG2D receptor, and activation markers. Our studies demonstrate that TCR γδ clonal leukemic blasts are more susceptible to lysis by effector γδ T cells over TCR αβ clonal leukemic blasts. Antigens expressed on TCR γδ clonal leukemic blasts specifically stimulate higher number of Vδ2 T cells from healthy PBMCs as compared to TCR αβ clonal leukemic blasts. TCR γδ clonal leukemic blasts showed significantly better immune synapse formation with γδ T cells over TCR αβ clonal leukemic blasts. Zoledronate treated TCR γδ clonal leukemic blasts show significantly higher F-Actin polarization and increased expression of CD166 and LFA-1 at the immune synapse with γδ T cells. The differences in effector immune cell composition and efficient lysis of TCR γδ clonal blasts explain the survival advantage of TCRγδ+T-ALL patients. The leukemic blasts in TCR γδ+T-ALL patients are able to form better immune synapse with immune cells such as γδ T cells, compared to TCRαβ+T-ALL patients. Figure 9.1 summarises the differences in molecules involved in the immune synapse formation of TCR γδ clonal and TCR αβ clonal leukemic blasts with γδ T cells.

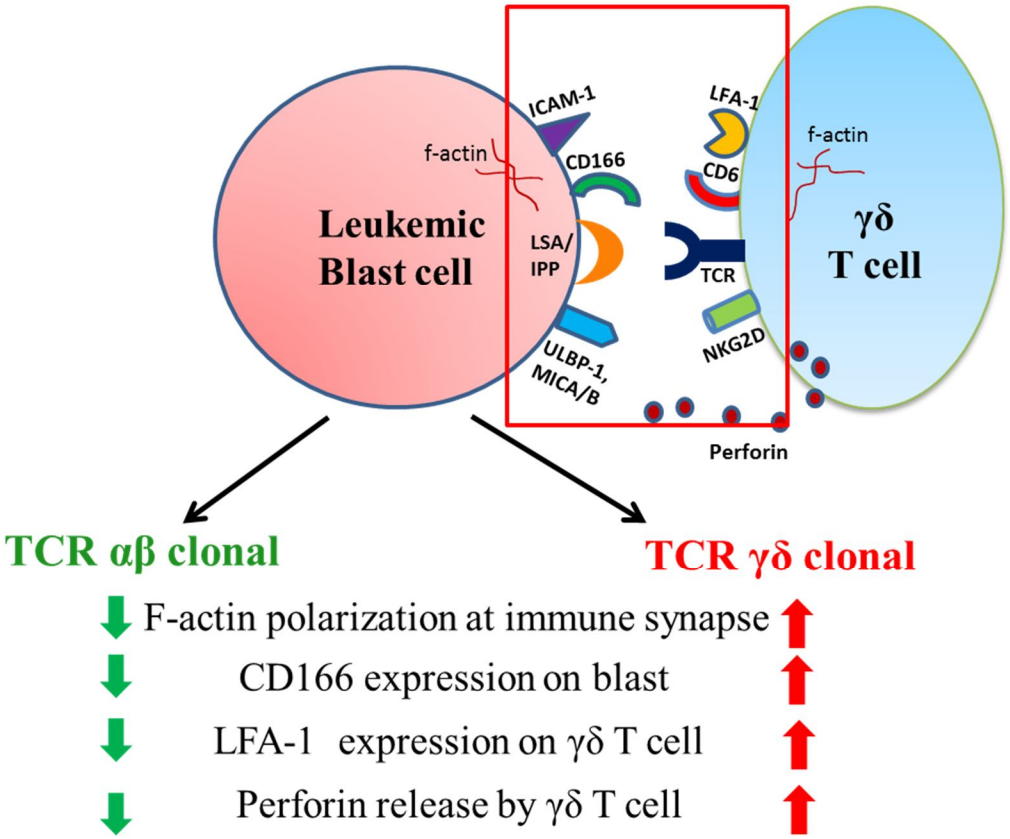


Figure 9.1 TCRγδ+T-ALL is a distinct subgroup from TCRαβ+T-ALL

We also studied the role of γδ T cells in ALL patients undergoing allogeneic BMT. Patients with earlier WBC engraftment and cGVHD post BMT, showed better overall survival and relapse free survival. Patients with earlier WBC engraftment (<15 days) showed distinct immune cell reconstitution than the patients with late WBC engraftment.

Recipients who achieved remission and who relapsed/died post BMT, showed distinct immune cell composition. Donor's immune cell composition affects the clinical outcome of recipients, and can be used as a predictor of disease outcome. If donors have higher Vδ1 TemRA and NKT cells there are more chances of recipients achieving remission. Cytomegalovirus (CMV) is a well-known cause of morbidity and mortality in bone marrow transplantation (BMT) recipients. We observed that recipients show significantly less CMV reactivation, if donors had high γδ T cells, CD19, CD4, Naïve Vδ2, Vδ2 TemRA, CD3/CD25, and Vδ2/CD25; and low CD3 EM, and Vδ1 EM cells. The clinical outcomes

influenced by different immune cell compositions in donors and recipients undergoing BMT are summarised in figure 9.2.

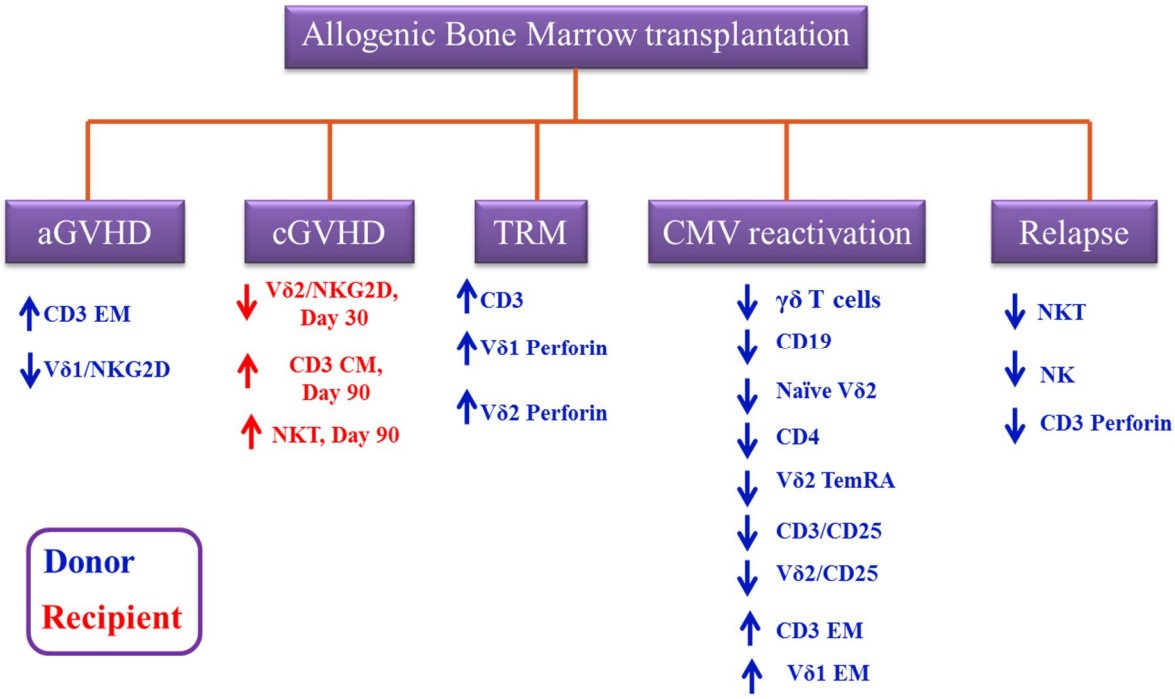


Figure 9.2 Immunophenotypes of donor and recipient influence clinical outcome post BMT

In conclusion, γδ T cells can be used as promising immunotherapeutic modality in T-ALL patients. Patients undergoing BMT can be administered with γδ T cell enriched grafts which may reduce the chances of GvHD and relapse and increase the graft versus leukemia effect.

In future TCR clonality can be used as a promising prognostic marker along with CDKN2A/CDKN2B CNV status of T-ALL patients. These prognostic markers would more efficiently risk categorise the T-ALL patients. Further genetic modification of γδ T cells with leukemia specific chimeric antigen receptors (CAR) can be considered as a potential immunotherapeutic approach in T-ALL patients.

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Publications

Leukemia Research 45 (2016) 33–39

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Research paper

Risk stratification of T-cell Acute Lymphoblastic Leukemia patients based on gene expression, mutations and copy number variation

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ARTICLE INFO

Article history:
Received 15 January 2016
Received in revised form 18 February 2016
Accepted 8 March 2016
Available online 1 April 2016

Keywords:
Gene expression profiling
T-ALL
Droplet digital PCR
Copy number variation
Mutations
TCRγδ + T-ALL

ABSTRACT

Gene expression, copy number variations (CNV), mutations and survival were studied to delineate TCRγδ + T-cell acute lymphoblastic leukemia (T-ALL) as a distinct subgroup from TCRαβ + T-ALL. Gene Ontology analysis showed that differential regulation of genes involved in pathways for leukemogenesis, apoptosis, cytokine-cytokine receptor interaction and antigen processing/presentation may offer a survival benefit to TCRγδ + T-ALL patients. Genes involved in disease biology and having equal expression in both the subgroups, were further analysed for mutations and CNV using droplet digital PCR. TCRγδ + T-ALL patients exhibited differential level of mutations for *NOTCH1* and *IKZF3*; however *BRAF* mutations were detected at equal levels in both the subgroups. Although TCRγδ + T-ALL patients with these mutations demonstrated improved disease-free survival (DFS) as compared TCRαβ + T-ALL patients, it was not statistically significant. Patients with homozygous deletion of *CDKN2A/CDKN2B* showed poor DFS in each subgroup. TCRγδ + T-ALL patients with wild type/heterozygous deletion of *CDKN2A/CDKN2B* possess significantly better DFS over TCRαβ + T-ALL patients ($p = 0.017$ and 0.045, respectively). Thus, the present study has for the first time demonstrated TCRγδ clonality and *CDKN2A/CDKN2B* CNV together as potential prognostic markers in management of T-ALL. Further understanding the functional significance of differentially regulated genes in T-ALL patients would aid in designing risk based treatment strategies in subset specific manner.

1. Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is a heterogeneous group of aggressive hematologic tumours resulting from the malignant transformation of T-cell progenitors arrested at various stages of development. T-ALL accounts for 10%–15% of paediatric and 25% of adult ALL cases and is characteristically more frequent in males than females [1]. T-ALL is a phenotypically and clinically heterogeneous disease. Major improvements have been made in ALL treatment, with successful long-term survival rates of approximately 80% over the past four decades. Despite the significant success rate, the remaining 20% of patients still present treatment failure [2]. The risk-based treatment assignment should be utilized to treat patients, who have a high frequency of treatment failure, which necessitates the availability of prognostic factors that reliably predict outcome [3].

In our earlier study on Indian T-ALL patients, we identified clonal TCR γ and δ junctional gene rearrangement status of T-ALL as a prognostic marker using a sensitive PCR-coupled Heteroduplex assay [4]. Clinically, survival probability was found to be significantly higher in TCRγδ + as compared to TCRαβ + T-ALL patients when γ and δ gene rearrangement was considered as prognostic variable [5,6]. This observation provided an impetus to investigate how TCRγδ + T-ALL differs biologically from TCRαβ + T-ALL subgroup.

Haydu and Ferrando [7] have reported activating Notch mutations and *CDKN2A/B* copy number variation (CNV) as potential prognostic markers in delineating T-ALL subgroup with poor prognosis. Whereas Moorman et al. [8] have delineated two ALL subgroups, exhibiting distinct treatment response, based on cyto-

Abbreviations: T-ALL, T-cell acute lymphoblastic leukemia; CNV, copy number variation; ddPCR, droplet digital PCR; OS, overall survival; DFS, disease free survival.

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http://dx.doi.org/10.1016/j.leukres.2016.03.002
0145-2126/

Table I
Characteristics of T-ALL patients.

Total number of T-ALL patients	n = 68
Age (years)	
Range	1–30
Median age	11
Gender	
Male: Female	59:9
Blast Count%	
Range	65–99
Median count	92
Absolute lymphocyte count ($x10^9$)	
Range	2.04–598
Median count	118.32
TCR Clonality	
TCRαβ clonal	38
TCRγδ clonal	30
Immunophenotypic Profile*	
CD2%	53.33
CD3%	16.66
CyCD3%	100
CD5%	71.42
CD7%	82.35
CD8%	25.8
CD34%	46.15

Abbreviations: T-ALL, T-cell acute lymphoblastic leukemia; TCR, T-cell receptor.
* % Positive patients for the marker.

genetic profile and CNV of *IKZF1, CDKN2A/B, PAX5, EBF1, ETV6, BTG1, RB1*, and *PAR1*. Mutations in *IKZF3, UBE2G2* and *ZEB2* were found to be of diagnostic importance in clinical management of leukemia other than T-ALL [9].

In the present investigation, we compared gene expression profile of TCRγδ + T-ALL versus TCRαβ + T-ALL subgroup to identify genes/pathways that provide survival advantage to TCRγδ + T-ALL patients. Further, we also analysed mutation and CNV in genes that were associated with survival (*NOTCH1, IKZF3, BRAF, CDKN2A/B* and *P53*). These markers may provide leads for further risk based stratification of treatment modalities.

2. Materials and methods

2.1. Subjects

Heparinized peripheral blood samples were collected from patients (n = 68) with newly diagnosed T-ALL, attending the outpatient department at Tata Memorial Hospital (TMH), Mumbai, India. The demographics and other details of patients have been compiled in Table I. Informed consent was obtained from all patients included in the study. Samples were collected during the period 2011 to 2014. The patients were later treated on modified MCP841 protocol (Supplemental material, Table A). The study was approved by Institutional Ethics Committee of Tata Memorial Centre, Mumbai. Leukemic blasts in peripheral blood of these patients ranged from 65 to 99%. Leukemic blasts were separated using ficoll-hypaque density gradient.

2.2. Sample preparation for microarray

Leukemic blasts from 14 out of 68 T-ALL patients (mentioned in Section 2.1) were used for gene expression profiling. Total RNA was isolated from leukemic blasts of TCRαβ + T-ALL (n = 9) and TCRγδ + T-ALL (n = 5) patients using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) as per the standard protocol. Samples with RNA Integrity Number (RIN) = 7.0 were used for gene expression profiling. Microarray experiments were performed through customized service using Agilent platform (Agilent Technologies, Palo Alto, CA, USA).

2.3. Microarray data analysis

Data was analysed using GeneSpring GX (v9.0) software (Agilent Technologies, Bengaluru, India) and TM4 Microarray Multiexperiment Viewer (T_MeV). Normalization of the data was done in GeneSpring GX using the recommended one color Per Chip and Per Gene Data Transformation. Significance Analysis of Microarray was also performed to identify differentially regulated genes. Differentially regulated genes were clustered using hierarchical clustering. Fold change was calculated as log base 2. p-value was calculated using volcano Plot.

2.4. Quantitative real time PCR for validation of gene expression

Microarray data was validated using quantitative real time PCR (qRT PCR). 30 out of total 68 T-ALL samples mentioned in section 2.1 were used for qRT PCR analysis. Relative mRNA level expression was quantitated using β-Actin (*ACTB*; NM_001101) as housekeeping gene. Fold changes in target gene expression were normalized to housekeeping gene via the published comparative $2^{-\Delta\Delta Ct}$ method [10].

2.5. Droplet digital PCR analysis for detection of mutation and copy number variation in selected genes

Droplet digital PCR (ddPCR) was carried out using the QX200 droplet digital PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). ddPCR analysis was performed on 50 out of 68 T-ALL patient samples (refer to Section 2.1) which had sufficient DNA quantity. Samples were analysed for mutations in *NOTCH1* (pL1600P), *IKZF3* (pL162R) and *BRAF* (pV600E) genes and CNV in *P53, CDKN2A* and *CDKN2B* genes. Droplet generation and droplet reading for ddPCR were carried out according to the manufacturer's instructions using Bio-Rad reagents. *RPP30* was used as reference gene in CNV analysis. Results were analyzed using QuantaSoft Software (Bio-Rad) and represented as copy numbers and concentration of mutation (copies/μl).

2.5. Statistical analysis

Graphs were plotted using Prism 5 software (GraphPad Software, Inc, CA, USA). Overall survival (OS) and disease free survival (DFS) were evaluated in TCRαβ + T-ALL and TCRγδ + T-ALL patients. Kaplan–Meier survival plots were generated for T-ALL patients based on mutations/CNV and TCR clonality using SPSS Graduate Pack 21.0 software (SPSS Inc, Chicago, IL, USA). P values were assessed using log-rank test. Survival analysis was performed with respect to multiple variables such as mutation, TCR clonality, and CNV. $P < 0.05$ was considered as statistically significant.

Detailed procedures are given in supplementary methods which is available online.

3. Results

Study was performed on 68 T-ALL patient samples, accrued from 2011 to 2014. 38 out of 68 patients (55.9%) were TCRαβ clonal and 30 out of 68 patients (44.1%) were TCRγδ clonal. Patients ranged in age from 1 to 30 years (median: 11 years). Blast count ranged from 65 to 99% (median: 92%). Median absolute lymphocyte count was 118.32×10^9 (Table I).

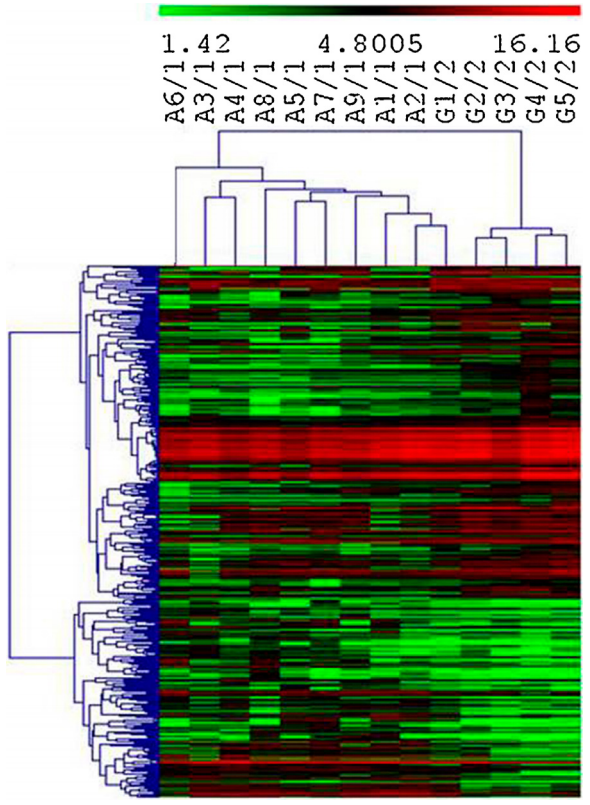

Fig. 1. Hierarchical clustering of T-ALL patients based on fold changes in gene expression.
Heatmap represents unsupervised clustering of 394 genes having 2 fold change in expression and p value <0.05 in T-ALL patients (n = 14). Columns represent two subsets of T-ALL patients (A1-A9, TCRαβ + T-ALL, n = 9; G1-G5, TCRγδ + T-ALL, n = 5) and each row represents a gene. Relative levels of gene expression are depicted with a colour scale, where red represents the high level of expression and green represents the low level.

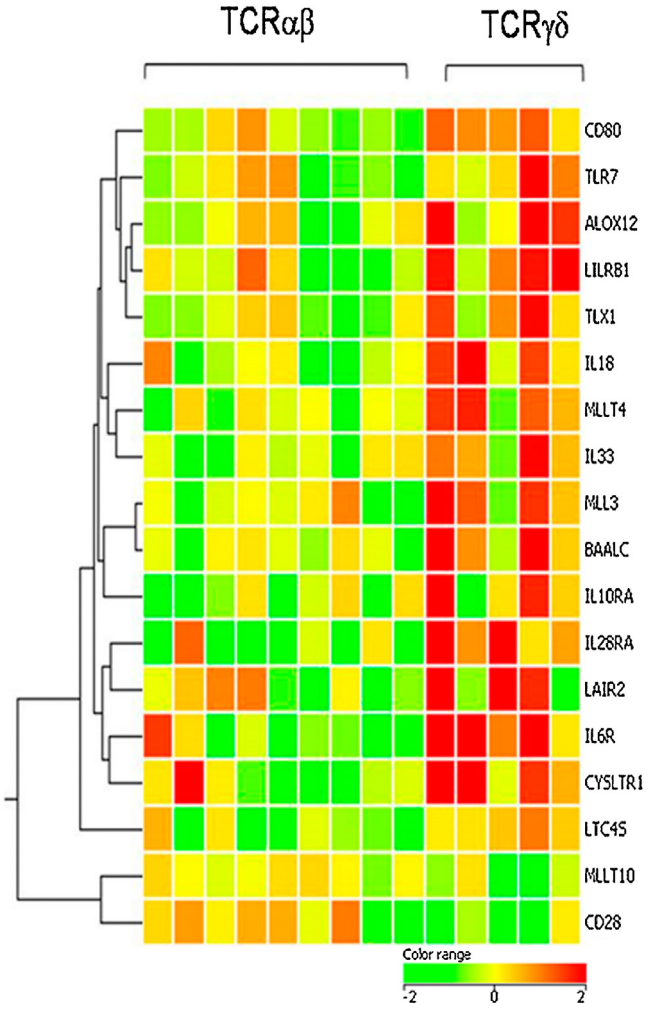

Fig. 2. Heatmap of leukemia associated genes in T-ALL patients.
Heatmap exhibits differential regulation of leukemia associated genes in TCRαβ + T-ALL (n = 9) V/s TCRγδ + T-ALL (n = 5) patients. These genes were selected on the basis of unsupervised clustering with non-average complete linkage rule and were statistically significant by unpaired students-t test ($p < 0.05$). Relative levels of gene expression are depicted with a colour scale, where red represents the high level of expression and green represents the low level.

3.1. Differential regulation of genes within two T-ALL subtypes

Unsupervised hierarchical tree distinguished two subsets of T-ALL patients i.e. TCRγδ+T-ALL and TCRαβ+T-ALL, showing differential regulation of genes based on 2 fold change in gene expression with $p < 0.05$ (Fig. 1). We identified list of 394 genes with 2 fold change and $p < 0.05$. Further, we could identify 3 clusters in these 394 genes, which are strongly correlated with each other within subgroups of T-ALL. These genes were found to be involved in the pathways related to cluster of differentiation, haematopoietic cell lineages, toll-like receptors, cytokine signalling molecules, inflammatory responses and mainly apoptotic pathway, as observed by functional annotation of genes using Gene Ontology, DAVID Bioinformatics Resources and KEGG database (Supplemental material, Table B). The data supports deregulation of genes in TCRαβ+T-ALL patients which are mainly involved in leukemogenesis. The gene expression data is deposited in NCBI's Gene Expression Omnibus (GEO, available at: http://www.ncbi.nlm.gov/geo/) and is accessible through GEO series accession number *GSE37389*.

3.2. Differential gene expression related to leukemia

Genes with statistical significant differential expression ($p < 0.05$) between TCRγδ + T-ALL and TCRαβ + T-ALL patients and categorised as "Leukemia associated genes" based on functions and pathways, using biological interpretation tool Biointerpreter (Genotypic Technology) and GeneSpring Software, were used to generate Heatmap (Fig. 2). CD28 and MLLT10 (Myeloid/lymphoid or mixed lineage leukemia) were significantly underexpressed leukemia related genes in TCRγδ+T-ALL patients. *IL6R, IL33, CD80, TLR7, ALOX12, LILRB1, TLX1, IL18, MLLT4, LTC45, MLL3, BAALC, IL10RA, IL28RA, LAIR2, CYSLTR1* were found to be significantly overexpressed in TCRγδ + T-ALL patients.

3.3. Gene expression profile related to apoptosis

Genes with statistical significant differential expression ($p < 0.05$) between TCRγδ + T-ALL and TCRαβ + T-ALL patients and categorised as "Apoptosis related genes" based on functions and pathways, using biological interpretation tool Biointerpreter (Genotypic Technology) and GeneSpring Software, were analysed further. *TNFRSF10C, FASLG, NFKB1* genes demonstrated significant fold change (1.34, 1.08 and −1.59 $\log_2$ respectively) between two subgroups. Heatmap was generated for a set of 23 apoptosis related genes that were significantly differentially regulated (Fig. 3). Out of these set of genes, 21 genes were up regulated in TCRγδ + T-ALL patients, while only 2 genes (SIAH1 and CD28) were down regulated. Increased expression of important genes such as

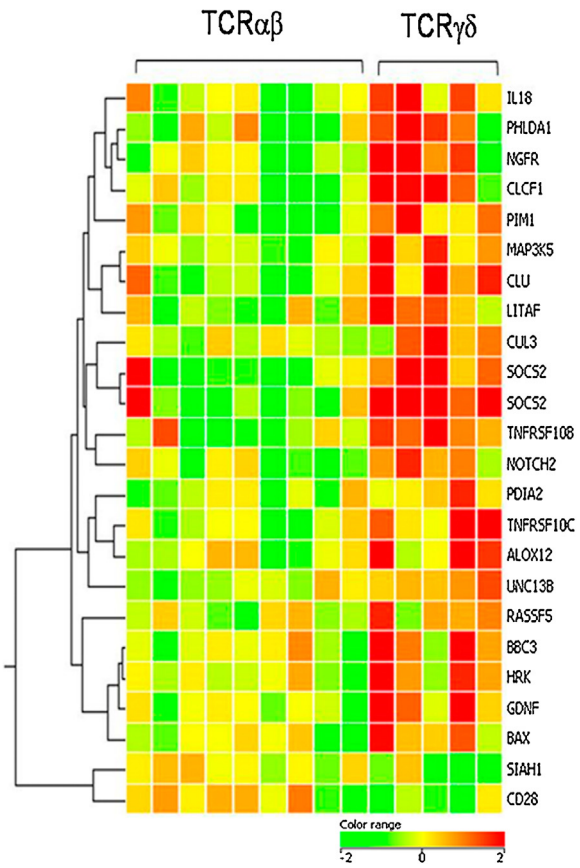

Fig. 3. Heatmap of apoptosis related genes in T-ALL patients.
Heatmap exhibits differential regulation of apoptosis related genes in TCRαβ + T-ALL (n = 9) V/s TCRγδ + T-ALL (n = 5) patients. These genes were selected on the basis of unsupervised clustering with non-average complete linkage rule and were statistically significant by unpaired students-t test ($p < 0.05$). Relative levels of gene expression are depicted with a colour scale, where red represents the high level of expression and green represents the low level.

TNFRSF10B, TNFRSF10C, PHLDA1, NGFR, BBC3, IL18, SOCS2, HRK, BAX was observed in TCRγδ+T-ALL patients.

3.4. Biological association of differentially regulated genes

Consistent with KEGG pathway analysis, based on 2 fold changes and $p < 0.05$, upregulated genes in TCRγδ + T-ALL patients belong to biological processes such as antigen processing and presentation (*IFI30, KLRD1, CD74, KIR2DS2*) and cell adhesion molecules (*HLA-DQB1, ICAM1, ITGA6, CD274*; Supplemental material, Table C). *TUBB6* and *PLCB4* of Gap Junction; *CACNA1H, HSPA1A, NFKB1* of MAPK signalling pathway; *IFNB1* and *CISH* of Jak-STAT signalling pathway were found to be down regulated in TCRγδ+T-ALL patients (Supplemental material, Table C).

3.5. Validation of gene expression by real time PCR

Based on degree of expression, fold regulation and involvement within signalling pathways (mapped by Natural Language Processing), 7 genes (*NFKB1, FASLG, HLA-DQB1, IFNB1, RXRA, CISH* and *SOCS2*) were shortlisted for further validation by qRT PCR. For six genes, qRT PCR data correlated very well with the microarray data (Fig. 4). It was observed that *IFNβ1* gene expression remain unchanged between two subgroups. Interestingly, mRNA expression of *RXRA* was found to be reversed between two subgroups as compared to microarray data. Out of these 7 genes, *FASLG, HLA-DQB1, SOCS2* were upregulated in TCRγδ + T-ALL (Fig. 4). Significant difference was observed in expression of *NFKB1* and *FASLG* between the two subgroups of T-ALL patients.

3.6. Comparative analysis of gene mutation In subgroups of T-ALL patients

Few important genes (*CDKN2A, CDKN2B, P53, NOTCH1, IKZF3* and *BRAF*) involved in survival/chemoresistance and whose gene expression levels were unaltered in TCRγδ + T-ALL and TCRαβ + T-ALL subgroups in microarray analysis, were selected to be further evaluated for differences in mutation or CNV.

2D amplitude plots were used to set threshold to determine presence or absence of mutations (Supplemental material, Fig. S1). TCRγδ+T-ALL patients exhibited variable frequency of mutations in *NOTCH1* and *IKZF3* as against TCRαβ + T-ALL patients, while BRAF mutations were detected at same level in both the subgroups (Fig. 5 A-C). DNA of all healthy individuals served as negative control and exhibited no mutation in any of the three genes studied.

Notch mutation was found to be higher in TCRαβ + T-ALL as compared to TCRγδ + T-ALL patients. 17 out of 26 (65.38%) TCRαβ + T-ALL patients showed mutations in Notch as compared to 13 out of 23 (56.52%) TCRγδ + T-ALL patients (Fig. 5A).

Mutation in *IKZF3* gene was detected at a low concentration (<0.5 mutant copies in a background of wild type DNA) in 11 of 22 (50%) TCRαβ+T-ALL patients and 8 of 19 (42.10%) TCRγδ + T-ALL patients (Fig. 5B). *IKZF3* mutation was found to be high in TCRαβ + T-ALL patients as compared to TCRγδ + T-ALL patients.

Concentration of BRAF mutation was also detected at very low level (<0.25 mutant copies) and number of patients showing the presence of mutation was equal in both the subgroups. 7 of 23 (30.43%) TCRαβ + T-ALL patients and 6 of 19 (31.57%) TCRγδ + T-ALL patients showed the presence of *BRAF* mutation (Fig. 5C).

3.7. Comparative analysis of gene copy number variations In subgroups of T-ALL patients

ddPCR CNV data of *CDKN2A, CDKN2B* and *p53* genes demonstrated distinct signatures of copy numbers in TCRγδ + T-ALL patients as against TCRαβ + T-ALL patients. DNA of healthy individuals served as negative control and exhibited wild type copy numbers of all the three genes (Fig. 5D–F).

In TCRαβ + T-ALL patients 17.39% (4 of 23) showed heterozygous deletion and 47.82% (11 of 23) showed homozygous deletion of *CDKN2A* (total deletion 65.21%, Fig. 5D). In TCRγδ + T-ALL patients 15.78% (3 of 19) showed heterozygous deletion and 52.63% (10 of 19) showed homozygous deletion of *CDKN2A* (total deletion 68.42%, Fig. 5D)

As shown in Fig. 5E, 50% TCRαβ + T-ALL patients showed deletion of *CDKN2B* copies, out of which 20.8% (5 of 24) showed heterozygous deletion and 7 of 24 (29.16%) showed homozygous deletion of *CDKN2B*. 63.15% TCRγδ + T-ALL patients showed deletion of *CDKN2B* copies, out of which 26.31% (5 of 19) showed heterozygous deletion and 36.84% (7 of 19) showed homozygous deletion of *CDKN2B*.

P53 copies were found to be deleted in only 1 patient out of 26 TCRαβ + T-ALL patients while there was no deletion of *P53* in any TCRγδ + T-ALL patient (Fig. 5F).

3.8. Correlation of mutation status with survival In T-ALL patients

Pairwise comparison of *BRAF, IKZF3* and *NOTCH1* mutation status and TCR clonality of T-ALL patients demonstrated differential survival of the two subgroups of patients viz. TCRαβ + T-ALL and TCRγδ + T-ALL. Based on presence or absence of gene muta-

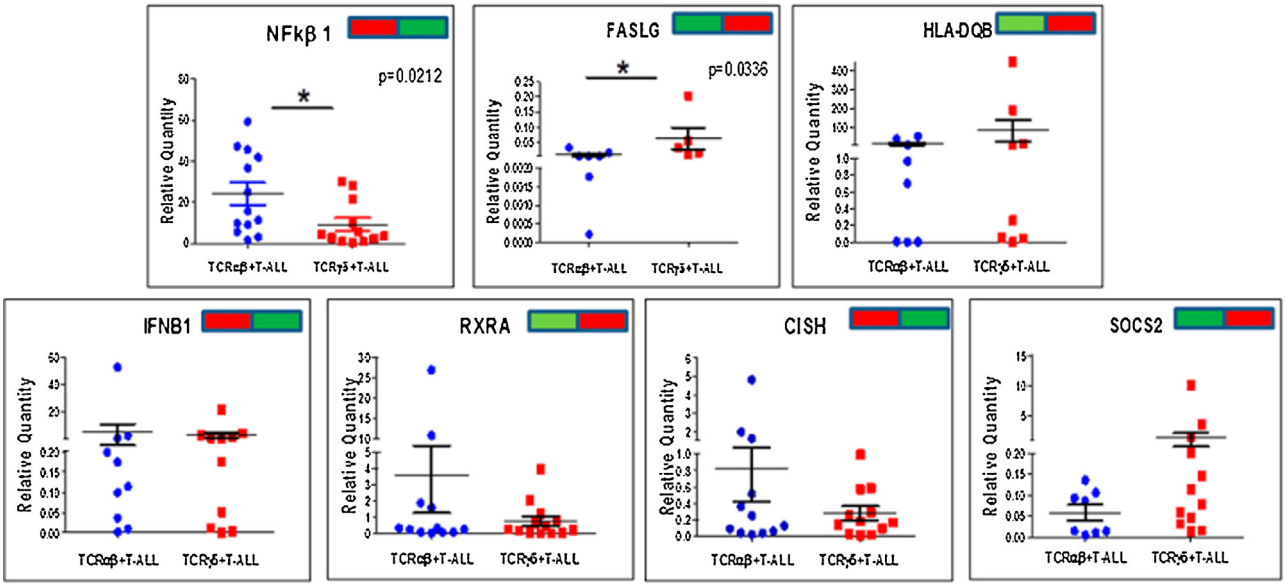

Fig. 4. qRT based validation of gene expression data in TCRαβ+T-ALL and TCRγδ + T-ALL patients.
Microarray data was validated for 7 genes using real time PCR technique. Genes such as NFKB1, IFNB1, RXRA, CISH were found to be expressed higher in TCRαβ + T-ALL patients (n = 13); while FASLG, HLA-DQB1 and SOCS2 were found to be expressed higher in TCRγδ + T-ALL patients (n = 17). The level of expression of these genes was consistent with microarray data. Upper right box in each panel indicates gene expression where red represents the high level of expression and green represents the low level. p value provided in the panel indicates statistical significance.

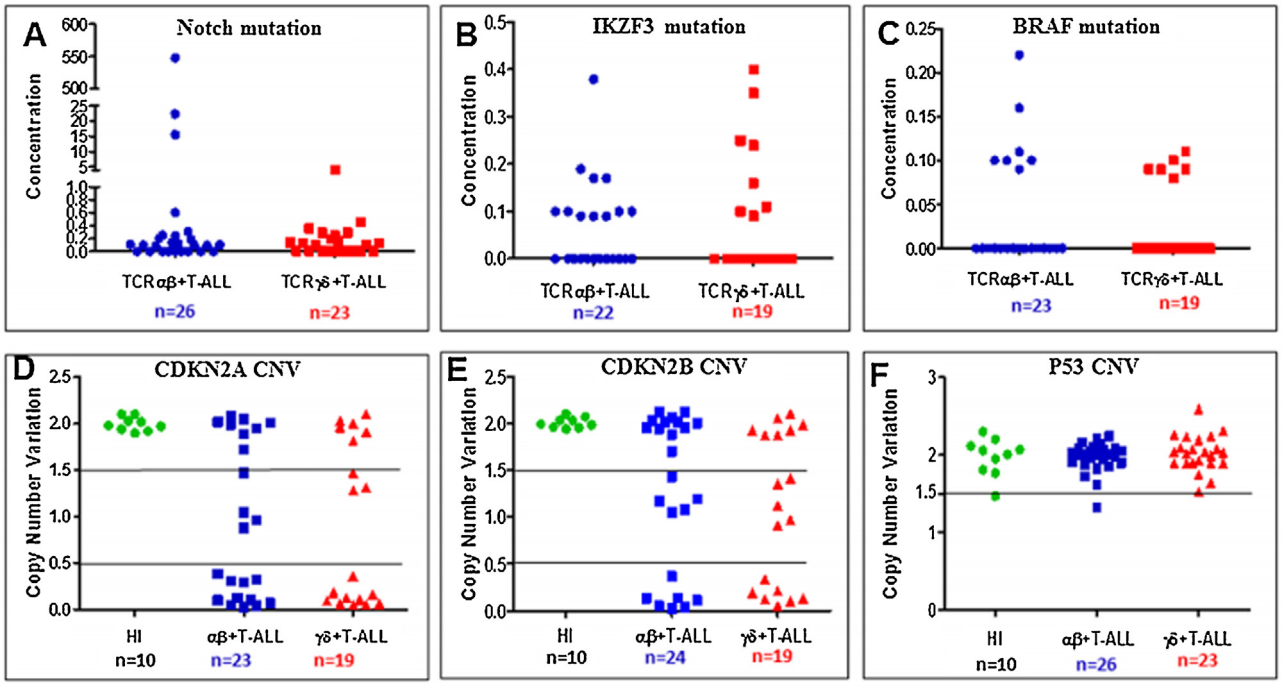

Fig. 5. Copy number variation and mutation analysis in T-ALL patients.
The scatter plots provide gene mutation analysis (panel A, B and C) and comparative CNV (panel D, E and F) in T-ALL patients. Panel A-C: comparative gene mutation analysis of Notch (A), IKZF3 (B) and BRAF (C) in TCRαβ + T-ALL as compared to TCRγδ + T-ALL patients. Panel D-F: comparative CNV of CDKN2A (D), CDKN2B (E) and P53 (F) in TCRαβ + T-ALL, TCRγδ + T-ALL and healthy individuals (HI). CNV range in each panel indicates homozygous deletion (CNV of 0–0.5), heterozygous deletion (CNV of 0.5–1.5) and wild type copies (CNV of 1.5–2.5). n values provided at bottom of each panel indicate number of patients used.

tion in *NOTCH1*, *IKZF3* and *BRAF*, the T-ALL patients (TCRαβ+ and TCRγδ+) showed differences in DFS (Supplemental material, Fig. S2). Although distinct trends were observed in survival, these were not statistically significant (P = 0.294, 0.509, and 0.156 for *NOTCH1*, *IKZF3* and *BRAF* respectively).

3.9. Correlation of CNV with survival In T-ALL patients

Pairwise comparisons of CDKN2A and CDKN2B CNV and TCR clonality in T-ALL patients (TCRαβ and TCRγδ) were performed. Correlation of DFS with *CDKN2A* and *CDKN2B* CNV and TCR clonality was found to be highly statistically significant (p = 0.017 and p = 0.045, respectively; Fig. 6A–B). In both subgroups of T-

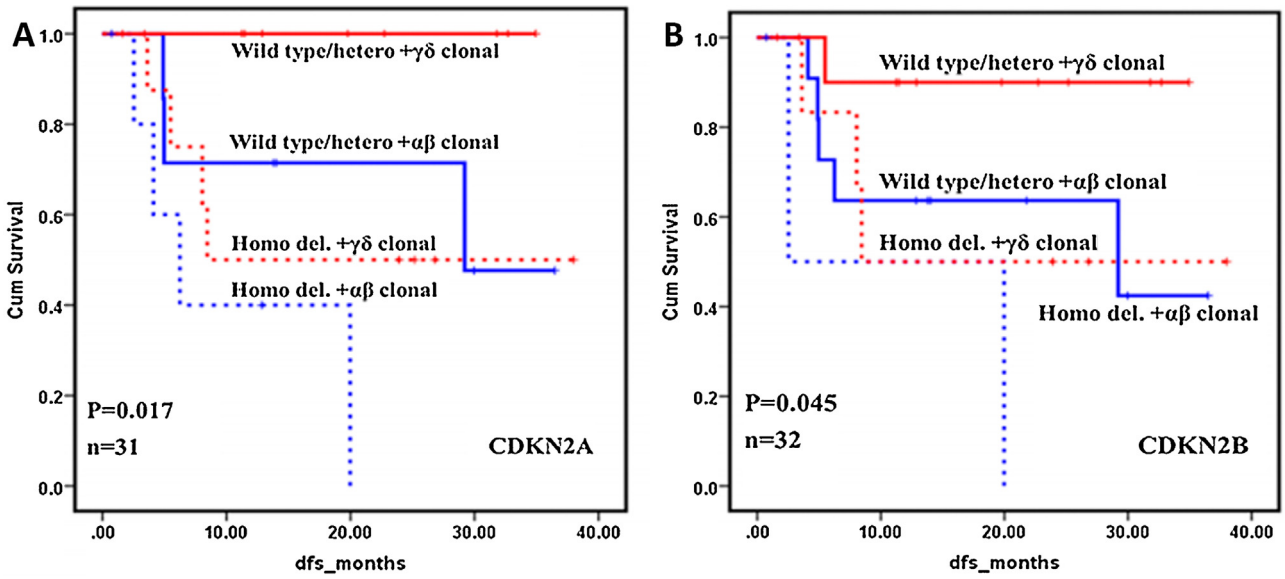

Fig. 6. Kaplan-Meier survival curves for T-ALL patients based on clonality status and CNVs.
The panels represent Kaplan-Meier survival curves for DFS of T-ALL subgroups. The parameters considered were T cell clonality and CDKN2A CNV (A); and CDKN2B CNV (B). P value provided in the panel indicates statistical significance and n indicates total number of patients included.

ALL, patients with wild type/heterozygous copies of CDKN2A/B showed improved disease free survival over patients with homozygous deletion of CDKN2A/B. In concordance with our previous studies [5,6], TCRαβ + T-ALL patients always showed poor DFS as compared to TCRγδ + T-ALL patients, even in presence of wild type/heterozygous copies of CDKN2A/B, (Fig. 6A–B). It was observed that *CDKN2A* with wild type/heterozygous deletion in TCRγδ + T-ALL patients showed significantly better OS over TCRαβ + T-ALL patients with homozygous deletion of CDKN2A (data not shown; p = 0.05); similarly TCRγδ + T-ALL patients with wild type/heterozygous deletion of CDKN2B, showed improved OS over TCRαβ + T-ALL, but was not statistically significant (data not shown). In conclusion, TCRγδ + T-ALL subgroup with wild type/heterozygous copies of CDKN2A/B showed significantly (p = 0.017 and p = 0.045, respectively) improved DFS as compared to TCRαβ + T-ALL.

4. Discussion

Our earlier studies have identified clonal TCR γ and δ junctional gene rearrangement status of T-ALL patients at diagnosis as a prognostic marker and predictor of response to chemotherapy. Our long term follow up study showed that TCRγδ + T-ALL patients have survival benefit, over TCRαβ + T-ALL patient cohort [4–6].

To unveil the reason behind the survival benefit to TCRγδ + T-ALL; in the present study we performed gene expression profiling as well as analysis of mutation and copy number variation in selected genes of these two T-ALL subsets. Our results have shown distinct signatures of TCRγδ + T-ALL and TCRαβ + T-ALL subgroups when compared with each other and also with normal lymphocyte profile.

NF-KB activation is important for the induction of T-cell leukemia, as suppression of the *NF-KB* pathway substantially reduces the severity of the disease [11]. In our study pro-survival *NFKB1* gene was found to be significantly upregulated in TCRαβ + T-ALL patients compared to TCRγδ + T-ALL subgroup. Other reports have demonstrated *NFKB1* activation to counteract apoptosis induced by TNF, Fas ligation, cytotoxic drugs, and other stimuli and block activation of caspase 8, a proteolytic enzyme involved in the apoptotic cascade [12].

Our results also show significant up regulation of survival related genes *BAX*, *HRK* and *FAS* in the TCRγδ + T-ALL patient subgroup. The susceptibility of cells to apoptosis depends on the relative expression of intracellular molecules that enhance apoptosis (pro-apoptotic), with BAX being the major representative molecule [13,14]. In accordance with our data, Ong et al. also observed that AML patients with high *BAX* expression at diagnosis exhibited better event-free survival and overall survival [15]. Further Sanz et al. demonstrated that *HRK* acts as a trigger for apoptosis and provokes cell death in hematopoietic progenitors even in the presence of survival factors [16]. While, in some paediatric ALL studies increased levels of *FAS* were shown to be associated with longer survival and complete remission [17].

Mutation and CNV analysis corroborated our earlier finding that TCRγδ + T-ALL is a distinct subgroup of T-ALL than TCRαβ + T-ALL. To the best of our knowledge, such an extensive analysis using clonality status, mutation/CNV and survival has not been reported earlier. Activating mutations in *NOTCH1* have been shown to occur in more than 50% of T-ALL cases [18]. We also observed similar results in our T-ALL cases when compared to normal lymphocytes. There are several conflicting reports on *NOTCH1* mutation and survival [19–21]. In the present study, association of survival with *NOTCH1* mutation was not statistically significant.

IKZF3 mutations have not been reported in T-ALL, but are reported in CML, B-ALL and B-CLL [22]. In our study, we found mutation in *IKZF3* gene at very low concentration in T-ALL patients. The importance of detecting *IKZF3* mutations in T-ALL lies in the fact that multiple therapeutic targets which are being investigated in patients with Ikaros mutation [23–25] could be of potential therapeutic benefit in patients with T-ALL.

Various previous reports have demonstrated that *BRAF* mutations are rare in hematologic malignancies including leukemias and lymphomas [26,27]. As we used highly precise and sensitive technique of ddPCR; we could detect very low level mutations in *BRAF* gene. Gustafsson et al. [28] reported no association between mutations in *BRAF* and DFS or any other clinical parameter. Similarly, we observed no significant difference in DFS based on BRAF mutation.

In our study, TCRγδ + T-ALL patients with wild type/heterozygous deletion of *CDKN2A/B* possess significantly better DFS over TCRαβ + T-ALL patients. While in both the

subgroups, homozygous deletion of *CDKN2A/B* showed inferior survival as compared to wild type/heterozygous copies, underscoring the importance of these tumour suppressor genes in Indian T-ALL cohort. The prognostic significance of CDKN2A deletions in ALL appears debatable. Vlierberghe et al. [19] have shown that homozygous deletion of CDKN2A/CDKN2B was associated with favorable outcome in T-ALL, whereas such correlation was not reported by others [29,30]. In accordance with our observations, deletion/methylation of *CDKN2A/B* was found to correlate with poor survival in Follicular Lymphoma patients [31], in Adult BCR-ABL1–Positive ALL [32] and in adult B-ALL [33]. Present study has reported for the first time, TCR clonality status in combination with CDKN2A/CDKN2B CNV to be associated with DFS in Indian T-ALL patients.

5. Conclusions

In conclusion, the present study has identified TCRγδ + T-ALL as a distinct subgroup from TCRαβ + T-ALL using gene expression profiling. Moreover this study has also identified CDKN2A/CDKN2B CNV combined with TCR clonality as potential prognostic marker in T-ALL patients.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors would like to thank Indian Council of Medical Research, Government of India for providing partial financial support for this project. Part of this work was also supported by Terry-Fox Foundation through Institutional Review Board, Tata Memorial Centre, Mumbai. Authors would also like to thank Dr P. Kondaiah, IISc, Bengaluru and Genotypic Technology Pvt. Ltd., Bengaluru, India, for the microarray processing and data analysis reported in this publication.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2016.03.002.

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