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Distinct sensitivity of $CD8^+CD4^-$ and $CD8^+CD4^+$ leukemic cell subpopulations to cyclophosphamide and rapamycin in Notch1-induced T-ALL mouse model

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ABSTRACT

The Notch1 signaling pathway plays an essential role in cell growth and differentiation. Over-expression of the intracellular Notch1 domain (ICN1) in murine hematopoietic cells is able to induce robust T-cell acute lymphoblastic leukemia (T-ALL) in mice. Here we explored the drug sensitivity of T-ALL cells in two subpopulations of $CD8^+CD4^+$ and $CD8^+CD4^-$ cells in Notch1-induced T-ALL mice. We found that Notch1 induced T-ALL cells could be decreased by chemotherapeutic drug cyclophosphamide (CTX). $CD8^+CD4^-$ T-ALL cells were more sensitive to CTX treatment than $CD8^+CD4^+$ T-ALL cells. The percentage of apoptotic cells induced by CTX treatment was higher in $CD8^+CD4^-$ T-ALL cells. T-ALL cells were also inhibited by inhibitor of mTORC1 rapamycin. $CD8^+CD4^+$ T-ALL cells were more susceptible to rapamycin treatment than $CD8^+CD4^-$ T-ALL cells. Rapamycin treatment selectively arrested more $CD8^+CD4^+$ T-ALL cells at G0 phase of cell cycle. A combination of the two drugs significantly improved overall survival of T-ALL bearing mice when compared with CTX or rapamycin alone. These results indicated that $CD8^+CD4^+$ and $CD8^+CD4^-$ leukemia cell populations had distinct drug sensitivity.

1. Introduction

T-ALL accounts for 25% of adult ALL and 10–15% of childhood ALL [1–3]. Although the survival rate of T-ALL patients has significantly improved with the advance of therapies, the outcome in adult relapsing T-ALL patients remains poor [4,5]. Several transcription factors, e.g., NOTCH1, LMO1/2, TAL1/2, HOX11, and Ikaros, have been implicated in the initiation and maintenance of T-ALL [6–9]. Among these, activating mutations of NOTCH1 have been observed in over 50% of T-ALL patients [10]. The NOTCH1 signaling pathway is evolutionarily conserved to regulate T cell growth and differentiation [11]. Notch1 gene encodes a conserved type I trans-membrane receptor, which is activated by the ligands of the Delta/Serrate/Lag-2 family expressed on the surface of neighboring cells. Once activated by ligand, the Notch1 receptors undergo proteolytic cleavage, release ICN1 from the plasma membrane, translocate to the nucleus to stimulate transcription of downstream target genes such as *hes1*, *hey1*, *c-Myc* and *cyclin D1* [12–14]. Activating mutation of Notch1 was frequently observed in the human T-ALL cell lines and T-ALL mouse models [15–17]. The potent oncogenicity of activating NOTCH1 mutation has been demonstrated in Notch1-induced T-ALL mouse model. Stable transduction of murine lineage negative (Lin^-) bone marrow (BM) cells with ICN1 can result in T-ALL development with 100% penetrance and this leukemia mouse model has been extensively used in the T-ALL studies [4].

c-MYC has been identified as a direct target gene of NOTCH1 during leukemogenesis [18,19]. PI3K-AKT-mTOR signaling pathway also plays an important role downstream of Notch1 signaling pathway. Inhibition of NOTCH1 signaling pathway suppresses mTOR signaling pathway in T-ALL cell lines [20]. NOTCH1 could facilitate the activation of PI3K/AKT/mTOR signaling pathway by down-regulating the expression of PTEN, which in turn is a critical negative regulator of PI3K/AKTmTOR signaling pathway [21]. PTEN posttranslational modification though phosphorylation and oxidation leads to activation of PI3K/AKT/mTOR signaling in T-ALL cells

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[22]. These results suggest that PI3K-AKT-mTOR signaling pathway plays an important role in T-ALL caused by activating NOTCH1 mutation and provided the rationale for the clinical use of mTOR inhibitors such as rapamycin and its analogs in T-ALL.

The chemotherapy regimen hyper-CVAD (fractionated CTX, vincristine, doxorubicin, dexamethasone) is effective for de novo ALL [23–26]. However, many T-ALL patients relapsed due to the development of resistance to CTX and other agents during the course of treatments. The drug resistance is partly due to the heterogeneity of T-ALL cells. The response of drug treatment in different populations of T-ALL cells is unclear. A common feature of heterogeneity to T-ALL is the presence of CD8 and CD4 double positive population ($CD8^+CD4^+$) as well as CD8 single positive population ($CD8^+CD4^-$) in many T-ALL mouse models, including Tal1/Lmo2 T-ALL mouse model, Ikaros-deficient T-ALL mouse model and ICN1 T-ALL mouse model [27–29]. To study the drug sensitivity of heterogeneous T-ALL cells *in vivo*, we used ICN1 T-ALL mouse model to examine the sensitivity of $CD8^+CD4^+$ and $CD8^+CD4^-$ T-ALL cells to the cell-cycle non-specific chemotherapeutic drug CTX and mTORC1 inhibitor rapamycin. We found that CTX induced more apoptosis in $CD8^+CD4^-$ T-ALL cells and rapamycin specifically inhibited cell cycle of $CD8^+CD4^+$ T-ALL cells. The results revealed higher sensitivity of $CD8^+CD4^-$ leukemia cells to CTX and higher sensitivity of $CD8^+CD4^+$ leukemia cells to rapamycin in a Notch1-induced T-ALL mouse model. We also demonstrated that combined use of CTX and rapamycin is more effective in reducing leukemia cells and prolonging T-ALL mice life span than either agent alone.

2. Materials and methods

2.1. Reagents and antibodies

Rapamycin (LC Laboratories) was dissolved in absolute ethanol (Sigma–Aldrich) to derive 10 mg/ml stock solution and stored at −80 °C. For *in vivo* experiments, rapamycin were prepared from the stock solution daily using sterile phosphate buffer solution (PBS) supplemented with 5% PEG-400 (Sigma–Aldrich) and 5% TWEEN-80 (Sigma–Aldrich). CTX (Sigma–Aldrich) was dissolved in sterile PBS to prepare a 40 mg/ml stock and stored at −80 °C. For *in vivo* experiments, CTX was diluted in sterile PBS to a final concentration of 4 mg/ml. PE conjugated CD3, PE-cy7 conjugated CD4, APC conjugated CD8, PE conjugated Phospho-Akt (Thr308) and PE conjugated Phospho-S6 (Ser244) were from BD Bioscience. PE conjugated Phospho-Akt (Ser473) was from Cell Signaling Technology.

2.2. Mice

Wild-type (WT) C57BL/6J mice were obtained from Jackson Laboratory. B6.SJL mice were purchased from Taconic Laboratory. For CTX treatment, 5×10^5 established ICN1 induced T-ALL cells were resuspended in 250 μl sterile PBS and transplanted into eight weeks old C57BL/6J mice through the tail vein. Mice were treated with CTX (50 mg/kg) or vehicle only once when the percentage of GFP^+ T-ALL cells in peripheral blood (PB) was greater than 10%. Different dosages of CTX had been tested in leukemia bearing mice and the dose of 50 mg/kg of CTX was chosen for the experiment to obtain suboptimal responses to the CTX treatment. The suboptimal dosage of CTX treatment allows a significant reduction of leukemic cells (instead of higher dosage to kill all leukemic cells) in order to observe and analyze the response of T-ALL cells upon CTX treatment. To test the effect of rapamycin on engraftment of T-ALL cells, 5×10^5 T-ALL cells were transplanted into WT mice. From the next day, these mice were treated with rapamycin at the dose of 1 mg/kg, 4 mg/kg, or 10 mg/kg by I.P. injection once per day for 14 days. To test the effect of rapamycin on proliferation of T-ALL cells, 5×10^5 T-ALL cells were transplanted into WT mice. These mice were treated with rapamycin (4 mg/kg) or vehicle once per day for 3 days or 10 days when the percentage of GFP^+ T-ALL cells in PB was greater than 10%. All mice were maintained under a specific pathogen-free facility. All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC), Institute of Hematology and Blood Disease Hospital, CAMS/PUMC. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.3. Establishment of ICN1 induced T-ALL mouse model

Transduction of primary BM cells was performed as previously described [30]. In brief, the plasmids (MSCV-ICN1-IRES-GFP, pKAT and pCMV-VSV-G) were co-transfected into packaging cell line 293T using lipofectamine 2000 (Invitrogen) to generate virus. Virus supernatants were harvested 48 and 72 h after transfection. Lin^- cells from the BM of B6.SJL mice ($CD45.1^+$) at the age of 8 weeks were enriched with Lineage Cell Depletion Kit (Miltenyi Biotec) according to the manufacturer's protocol. The Lin^- cells were then transduced with viruses and the transduction efficiency was measured by flow cytometry. Bone marrow nucleated cells (BMNCs, 10^6/host) from C57BL/6J mice ($CD45.2^+$) at the age of 8 weeks were transplanted into lethally irradiated (9.5 Gy) C57BL/6J mice with 10^6 ICN1 virus-transduced Lin^- cells from B6.SJL mice as indicated in Fig. 1A. Primary $CD45.1^+$ GFP^+ T-ALL cells were isolated from BM and spleen of end-stage primary leukemia mice and transplanted into sub-lethal irradiated (4.8 Gy) recipient mice. T-ALL cells were collected from the BM and spleen of the end-stage recipient mice and stored. Collected T-ALL cells were subsequently engrafted in WT mice to induce T-ALL for CTX and rapamycin treatment.

2.4. Flow cytometry analysis

Mouse BM single-cell suspension was obtained by flushing ilias, femurs, and tibias as described [31]. GFP^+ T-ALL cells in PB, BM and spleen were detected by flow cytometry. To determine the immune-phenotypes of T-ALL cells, BM cells of T-ALL mice were labeled with antibodies against CD4 and CD8. For protein kinase phosphorylation analysis, BM cells of T-ALL mice were labeled with surface antibodies against CD4 and CD8, fixed and permeabilized with BD IntraSure Kit (BD Bioscience), and then intracellularly stained with antibodies against p-S6 (pS244), p-AKT (pT308), or p-AKT (pS473). For cell-cycle analysis, BM cells of T-ALL mice were labeled with CD4 and CD8 antibodies, fixed and permeabilized with BD IntraSure Kit (BD Bioscience), and then intracellularly stained with antibody against PE conjugated Ki67 and Hoechst 33342 (BD Bioscience). For apoptosis assay, BM cells of T-ALL mice labeled with CD4 and CD8 antibodies were stained with PE conjugated Annexin V and 7-AAD (BD Bioscience). Analyses were performed on a LSR II (BD Bioscience).

2.5. RNA extraction and quantitative RT-PCR

BM single-cell suspension from T-ALL mice was labeled with antibodies against CD8 and CD4. A total of 5×10^5 $CD8^+CD4^+$ and $CD8^+CD4^-$ GFP^+ T-ALL cells were sorted with BD FACSAria III (BD Bioscience). Total RNA was extracted with an RNeasy Mini Kit (QIAGEN). Reverse transcription was done using $Oligo(dT)_{18}$, 2 × TS Reaction Mix, and TransScript RT/RI Enzyme Mix (Transgen). RT-PCR was done with FastStart Universal SYBR Green Master (Roche), 0.4 μM of specific forward and reverse primers, and normalized cDNA. The parameters for the thermal cycling of PCR were as follows: 15 s at 95 °C and 60 s at 60 °C, 40 cycles. All the primer sequences are listed Supplementary Table 1.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2013.09.007.

2.6. Statistical analysis

Student's *t* tests were used for comparisons between 2 groups and ANOVA analysis for multiple groups. The Kaplan–Meier method was used to construct survival curves, and survival results were compared using the Mantel–Cox log-rank test. All results represent the average of at least three independent experiments and are expressed as mean ± SD. $P < 0.05$ was considered to be significantly.

3. Results

3.1. $CD8^+CD4^+$ and $CD8^+CD4^-$ T-ALL cells are equi-potent in T-ALL induction in WT mice

We established the mouse T-ALL model in which lethally irradiated recipients (C57BL/6J) were transplanted with 10^6 cells that were transduced with the ICN1 expression viruses (starting with Lin^- cells) from B6.SJL mice ($CD45.1^+$) and 10^6 BMNCs from C57BL/6J mice ($CD45.2^+$) (Fig. 1A). After 2 to 4 weeks transplantation, PB and BM are dominated by $CD45.1^+$ GFP^+ T-ALL cells. We have observed that all the GFP^+ T-ALL cells co-expressed CD45.1, indicating that all the GFP^+ T-ALL cells were from the donor cells of B6.SJL mice. In the subsequent experiment, we used GFP as the marker to distinguish donor T-ALL cells from the recipient mice. Six weeks after transplantation, gross examination of tissues from the sick mice revealed hepatomegaly, splenomegaly and lymphadenopathy resulting from extensive organ infiltration by lymphoblasts. 100% of the recipient mice that received Notch1-overexpressing cells developed T-ALL within 8 weeks. Primary GFP^+ T-ALL cells from three end-stage leukemia mice were harvested and then transplanted into sub-lethally recipients for expansion. Transplantation of isolated T-ALL cells into un-treated WT mice