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# Cellular proteolytic modification of tumor-suppressor CYLD is critical for the initiation of human T-cell acute lymphoblastic leukemia

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ABSTRACT

There exists a general recognition of the fact that post translational modification of CYLD protein through proteolytic cleavage by MALT-1 results in sustained cellular NF-kB activity which is conspicuously found to be associated with cancer in general and hematological malignancies in particular. The present study was directed to understand the contribution of MALT-1 and deubiquitinase CYLD to the initiation of T-cell acute lymphoblastic leukemia (T-ALL). Such a study revealed for the first time that the 35 kDa CYLD cleaved factor generated by MALT-1 mediated proteolytic cleavage was conspicuously present in human T- ALL subjects of pediatric age group. Further, over-expression of this 35 kDa CYLD factor within normal human peripheral blood mononuclear cells had the inherent capacity to program the genome of these cells resulting in T-cell lineage ALL. Based upon these results, we propose that MALT1 inhibitors may be of crucial importance in the treatment of T-ALL subjects of pediatric age group.



## Introduction

Several studies have demonstrated a crucial involvement of both canonical and non-canonical NFkB activation in the evolution of human hematological malignancies [1]. Such NFkB pathways have been found downstream of oncogenic Notch-I responsible for thymocyte neoplastic disease known as T-cell acute lymphoblastic leukemia [1,2]. It has been recently demonstrated that Notch through Hes 1, a canonical Notch target and transcriptional repressor, sustains NFkB activation by repressing the deubiquitinase CYLD [2]. Cylindromatosis (CYLD), a tumor suppressor gene, has been shown to regulate the activity of NFkB within human cells. Post-translational modifications of CYLD including phosphorylation, ubiquitination and proteolytic cleavage appear to be critical for its function [3–5]. The para-caspase mucosa associated lymphoid tissue (MALT-1) has recently emerged as the most critical regulator of CYLD-mediated NFkB activation in various immune and non-immune cells [6–8]. In this context, the present study was addressed to understand three specific issues: 1) how MALT-1 over-expression within human blood mononuclear cells programs these cells?; 2) does MALT-1 induced CYLD-cleavage govern this transformation?; and 3) does similar CYLD post-translational modification occur in T-cells derived from acute lymphoblastic leukemia subjects of pediatric age-group?

## Material and methods

### *Cell culture*

Normal peripheral blood mononuclear cells (PBMCs) were isolated from blood withdrawn from 15 normal healthy volunteers (with their prior informed consent ensuring that these subjects had abstained from any medication for 2 weeks before blood donation) using density gradient centrifugation method and cultured as described before [9]. T-cells were isolated by MACS separation according to the manufacturer's protocol (Miltenyi Biotec, Germany). Freshly diagnosed T-ALL patient subjects of pediatric age group ($n = 15$) were employed from the outpatient department (OPD) of Pediatrics of our institute with their prior informed consent and ethical approval by institute's ethical committee. Further, the study conforms to the principles outlined in the declaration of Helsinki [10].

### *Transfection*

p3F-Strep-mMALT1 obtained from "Addgene plasmid 33315" [11] was transfected into PBMCs using ESCORT transfection reagent (Sigma) to overexpress MALT1 protein. siRNA against conserved sequence of MALT1 mRNA (Sigma) was used to knock-down the expression of MALT1. The sequences corresponding to the MALT1 cleaved CYLD—35 kDa and 70 kDa, were amplified using specific designed primers and the PCR amplicons were cloned in a pEF6V5His TOPO expression vector

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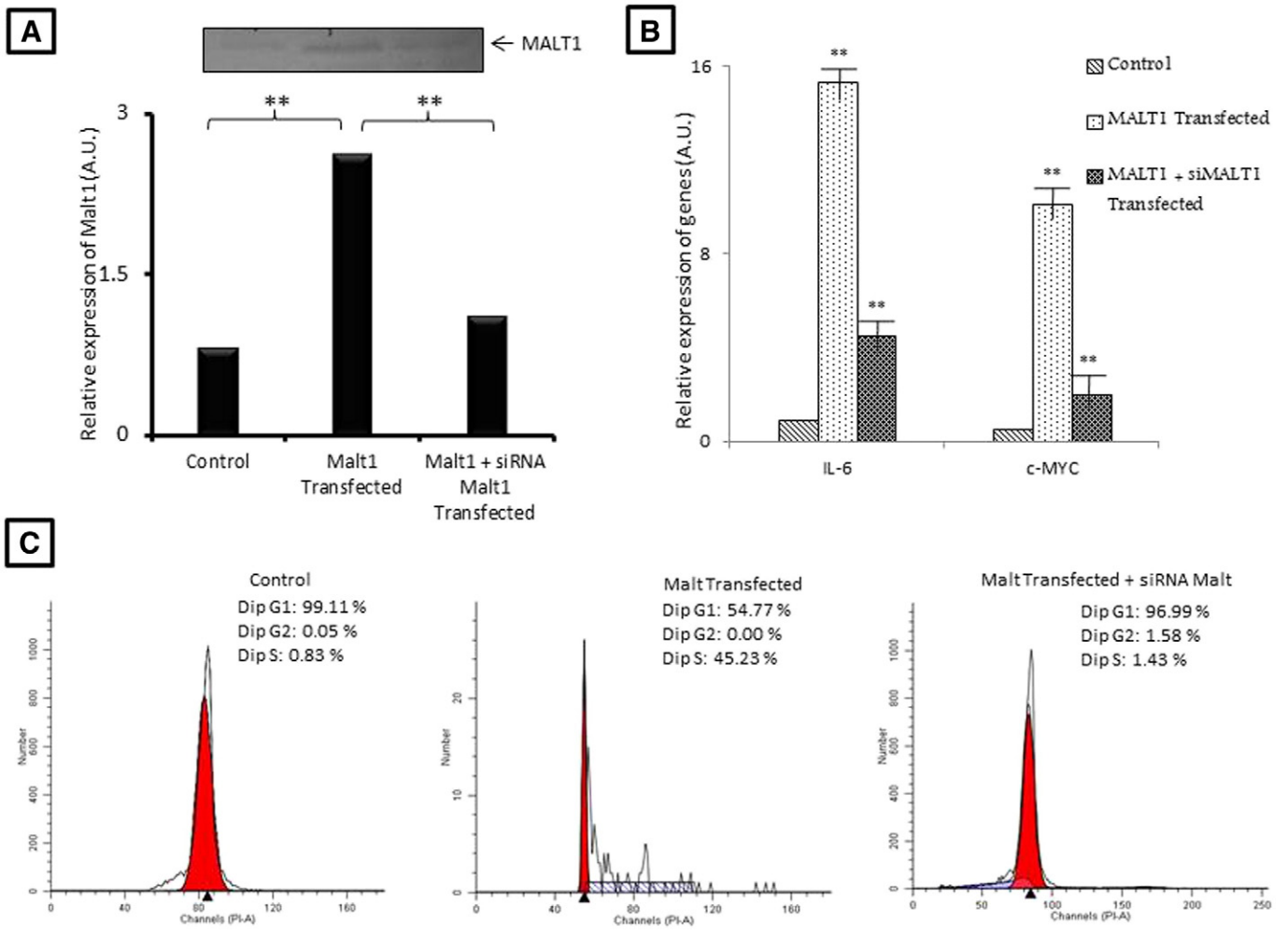


**Fig. 1.** MALT1 dependent transformation of normal human PBMCs: Ectopic MALT1 over-expression using a specific MALT1 expression plasmid & its suppression using a specific siRNA against MALT1 mRNA within normal human PBMCs showed significant increase and decrease in the expression of MALT1 gene product respectively (A). (B) Cellular MALT1 overexpression results in increased expression of IL-6 (markers of NF-kB activity) and C-myc (marker of cellular proliferation). The expression of MALT1 positively regulates the entry of normal PBMCs into cell cycle progression (C).

(Invitrogen) and subsequently transfected into normal human PBMCs followed by maintenance of these cells in vitro culture up to 5 days.

*Expression analysis*

Transcriptional expression of various genes was analyzed by RT-PCR using gene specific primers. Further the PCR products were run on 2.5% agarose gel and to analyze the relative expression of genes a densitometry scanning of bands was done using scion image analysis software.

*Immuno-blot analysis and immuno-precipitation*

Cellular protein was extracted and subsequently subjected to SDS-PAGE, followed by western blotting and immuno-detection as per the standard procedure [9] using specific antibodies against MALT1, CYLD (Polyclonal and C terminal) and β-actin obtained from Sigma. Cellular immuno-precipitation experiments were done as described previously [9]. In order to verify the status of NFkB activity, the PBMCs transfected with 35 kDa factor were processed for separation of cytoplasmic and nuclear protein fractions. The cells were suspended in buffer containing 250 mM Sucrose, 20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and protease inhibitor cocktail (Sigma) passed through a 25G needle 10 times using a 1 ml syringe and left on ice for 20 min. The nuclear pellet was centrifuged out, washed with buffer and re-suspended in standard lysis buffer with 10% glycerol and 0.1% SDS. The cytoplasmic extract was subjected to ethanol precipitation and further re-suspended in standard lysis buffer. These extracts were immuno-blotted using primary antibodies against Rel A/p65 (Sigma), pIKBα (Cell Signaling Technologies). β-Actin (Sigma) and histone H3 (Sigma) were used as invariant controls for cytoplasmic and nuclear protein fractions respectively.

*Flow cytometry*

Cells were incubated with combinations of antibodies to cell surface determinants, conjugated to PE, FITC, Cy-Cy-chrome, or biotin. Antibodies specific to the following surface markers were purchased from BD Biosciences: CD1A, CD2, CD3, CD4, CD5, CD8, CD10, CD13, CD20, CD33, CD34, CD38, CD79a, HLADR, and TdT. Biotinylated cells were visualized using streptavidin conjugated to PE or Cy-Cychrome (BD Biosciences). All samples were acquired on BD LSR II (BD Biosciences), and results were analyzed with BD FACSDiva v6 Software (BD Biosciences). Absolute numbers of lymphocyte subpopulations were calculated based on their percentage and the total number of lymphocytes. For cell cycle analysis, cells were permeabilized using 70% ethanol for 2 h and incubated with RNase A at 37 °C for 15 min. Cells were acquired on BD LSR II (BD Biosciences) immediately after staining with propidium iodide (PI). Apoptosis was checked using Annexin V/PI kit (Sigma).