Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.13019

Exonal switch down-regulates the expression of CD5 on blasts of acute T cell leukaemia

A. K. Rai ,*† A. Singh,*¶ A. Saxena,* ** T. Seth,‡ V. Raina§†† and D. K. Mitra *

*Cellular Immunology Division, Department of Transplant Immunology and Immunogenetics, All India Institute of Medical Sciences (AIIMS), New Delhi, India, †Department of Biotechnology, Motilal Nehru National Institute of Technology Allahabad, Allahabad, India, ‡Departments of Hematology, and §Medical Oncology, BRAIRCH, All India Institute of Medical Sciences (AIIMS), New Delhi, India

Accepted for publication 23 July 2017
Correspondence: D. K. Mitra, Professor, Cellular Immunology Division, Department of Transplant Immunology and Immunogenetics, All India Institute of Medical Sciences (AIIMS), Ansari Nagar, New Delhi 110029, India.
E-mail: salilmitra2@gmail.com and A. K. Rai, Assistant Professor, Department of Biotechnology, Motilal Nehru National Institute of Technology, Allahabad 211004, Uttar Pradesh, India.
E-mail: ambakrai@mnnit.ac.in

Present Addresses: ¶Division of Transplantation, Department of Surgery, Schulze Diabetes Institute (SDI), University of Minnesota, Masonic Memorial Building, Minneapolis, MN, USA

**National Heart, Lung, and Blood Institute (NHLBI), National Institute of Health (NIH), Bethesda, MD, USA

††Medical Oncology Haematology and BMT Fortis Memorial Research Institute, Gurgaon, India

Summary

To date, CD5 expression and its role in acute T cell lymphoblastic leukaemia (T-ALL) have not been studied closely. We observed a significant reduction in surface expression of CD5 (sCD5) on leukaemic T cells compared to autologous non-leukaemic T cells. In this study, we have shown the molecular mechanism regulating the expression and function of CD5 on leukaemic T cells. A total of 250 patients suffering from leukaemia and lymphoma were immunophenotyped. Final diagnosis was based on their clinical presentation, morphological data and flow cytometry-based immunophenotyping. Thirty-nine patients were found to be of ALL-T origin. Amplification of early region of E1A and E1B transcripts of *CD5* was correlated with the levels of surface and intracellular expression of CD5 protein. Functional studies were performed to show the effect of CD5 blocking on interleukin IL-2 production and survival of leukaemic and non-leukaemic cells. Lack of expression of sCD5 on T-ALL blasts was correlated closely with predominant transcription of exon E1B and significant loss of exon E1A of the *CD5* gene, which is associated with surface expression of CD5 on lymphocytes. High expression of E1B also correlates with increased expression of cytoplasmic CD5 (cCD5) among leukaemic T cells. Interestingly, we observed a significant increase in the production of IL-2 by non-leukaemic T cells upon CD5 blocking, leading possibly to their increased survival at 48 h. Our study provides understanding of the regulation of CD5 expression on leukaemic T cells, and may help in understanding the molecular mechanism of CD5 down-regulation.

Keywords: acute leukaemia, CD5, down-regulation, immune suppression, T cell

Introduction

The biology of T cell acute lymphoblastic leukaemia (T-ALL) is complex, and remains incompletely understood [1,2]. Delineating critical molecular event(s) underlying the leukaemic transformation of T cells is important not only for understanding the pathology, but also for identifying putative therapeutic target(s). Upon entering the thymus, precursor T cells undergo differentiation through negative and positive selection to emerge as fully matured and competent T cells [3]. Pathogenesis of T-ALL involve disruption of this process, resulting in multiple oncogenic changes leading to differential protein expression that alter key regulatory processes in the cell [4]. This

eventually subverts the normal control of thymocyte homeostasis, favouring leukaemic transformation.

CD5 is a transmembrane 67-kDa surface glycoprotein predominantly expressed on thymocytes, peripheral mature T cells and a subset of B cells (B1a cells). It has been described as a key regulatory protein involved in controlling the T cell receptor-mediated activation of T cells [5,6]. CD5 is thought to function as a negative regulator of the T cell receptor (TCR) activation signal, as the absence of CD5 skews the developing T cells towards uncontrolled TCR/CD3-mediated proliferation [7–11].

B1 cells, a mature subset of B cells, are divided further into two subsets, B1a and B1b, depending on the presence and absence of surface (sCD5), as well as intracellular (cCD5) CD5 expression. B1a cells express sCD5 while B1b cells do not, but demonstrate cCD5 [5,12,13]. Expression and intracellular localization of CD5 in these B cell subsets is regulated by exonal switching of alternatively spliced variants of CD5 gene transcripts, E1A and E1B, respectively [14,15]. The former encodes CD5 protein with a leader peptide that enables its cell surface expression. Conversely, CD5 protein encoded by the latter lacks leader peptide and fails to reach the cell surface, resulting in an intracellular accumulation in B1b cells [14,15]. Differential transcription of these isoforms of a single CD5 gene in B cell subsets (B1a and B1b) highlights the relevance of this exonal switching in the cellular localization of CD5 molecules.

Emphasizing its negative regulatory effect, the role of CD5 in the process of neoplastic transformation of lymphocytes remains unclear. However, its role in the anti-tumour immune response is shown[16]. The varied expression and localization (surface/intracellular) of CD5 between two subsets of B cells and its negative regulatory role encouraged us to investigate the expression of CD5 among T-ALL patients [17–21]. We hypothesized that variation in CD5 expression and localization due to exonal switching may be involved in the leukaemic transformation of T cells. In the present study, we measured the levels of expression of CD5 on normal and leukaemic T cells of T-ALL patients. In order to understand the mechanism of CD5 expression in T-ALL cases, we also measured the expression of exons E1A and E1B. The same was linked to the levels of surface and cytoplasmic CD5 protein (sCD5 and cCD5) in healthy and leukaemic T cells. Upon CD5 blocking, we found restoration of the normal compartment of T cells in terms of proportional increase and interleukin-2 (IL-2) production. Importantly, there was a simultaneous decrease in the number of leukaemic T cells.

Materials and methods

Patients, their samples and immunophenotyping

In total, 250 patients were immunophenotyped, which comprised patients suffering from acute lymphoid/myeloid leukaemia (ALL/AML), non-Hodgkin's lymphoma (NHL), biphenotypical, bi-lineage and chronic lymphoproliferative disorders (CLPD). All cases of acute leukaemias, with no prior treatment with steroids or any other form of chemotherapy, were included into the study. Patients with diagnoses of chronic myeloid leukaemia-blast crisis, relapsed leukaemia and secondary leukaemia were excluded from the study. Informed consent was obtained. Samples were collected in heparinized tubes (BD Vacutainer^{TM} sodium heparin, cat. no. 366480; Becton Dickinson, Franklin Lakes, NJ, USA) as part of the leukaemia phenotyping patient service carried out at the Department of Transplant Immunology and Immunogenetics, AIIMS, New Delhi. Ethical approval was obtained from the Ethics Committee (ref. no. 278/R&C/14-15, dated 17/12/2014). A battery of antibodies against lineage-specific and/or associated markers were used to immunophenotype the cells of different origin, as per the modified European Group for the Immunological Classification of Leukaemias (EGIL)[23] [tumour *versus* non-tumour: CD45; T-ALL: cCD3, CD5 and CD7; B-ALL: CD19, CD10, CD20 and CD22; AML: myeloperoxidase (MPO), CD13 and CD33; other markers (optional): CD34, CD38, terminal deoxynucleotidyl (TdT), CD2 and human leucocyte antigen D-related (HLA-DR), etc.; Fig. 1a–d]. Final diagnosis was based on clinical presentation, morphology and fluorescence activated cell sorter (FACS)-based immunophenotyping. Experiments were performed only in cases where leftover cells were sufficient in number. Finally, 39 patients [age, mean ± standard deviation (s.d.), 23·27 ± 14·57; male/female, 30/9] were found to be of ALL-T origin. Their specimens were mainly bone marrow ($n = 26$) and peripheral blood ($n = 13$) and subjected for molecular analysis irrespective of their CD5 expression. Patients' details are given in Table 1.

Reagents

RPMI medium (Caisson Laboratories, Smithfield, UT, USA; cat. no.: 010P, with L-glutamine, without HEPES and $NaHCO_3$) were used as a nutrient medium, supplemented with 10% fetal calf serum (FCS) (Biological Industries, Kibbutz Beit Haemek, Israel) for proper maintenance of cells. L-glutamine (G-5763; Sigma-Aldrich, St Louis, MO, USA) and antibiotics (Pen-Strep-Ampho Sol; Biological Industries) were added as a supplement to RPMI. Monoclonal antibodies used were fluorescein isothiocyanate (FITC)-conjugated CD45 (clone: HI30, cat. no. 555482), CD7 (clone: M-T701, cat. no. 555360), CD22 (clone: HIB22, cat. no. 555424), HLA-DR (clone: G46–6, cat. no. 555811, all BD Pharmingen, San Diego, CA, USA), MPO (clone: 2C7, MCA1757F; Serotec, Oxford, UK), immunoglobulin M (IgM) (clone: IIE2, prod. no. 1031; Diatec, Oslo, Norway), CD14 (clone: 18D11, prod. no. 3111; Diatec); phycoerythrin (PE)-conjugated CD3 (clone: HIT3a, cat. no. 555340), CD5 (clone: UCHT2, cat. no. 555353), CD10 (clone: HI10a, cat. no. 555375), CD13 (clone: WM15, cat. no. 555394), CD33 (clone: WM53, cat. no.

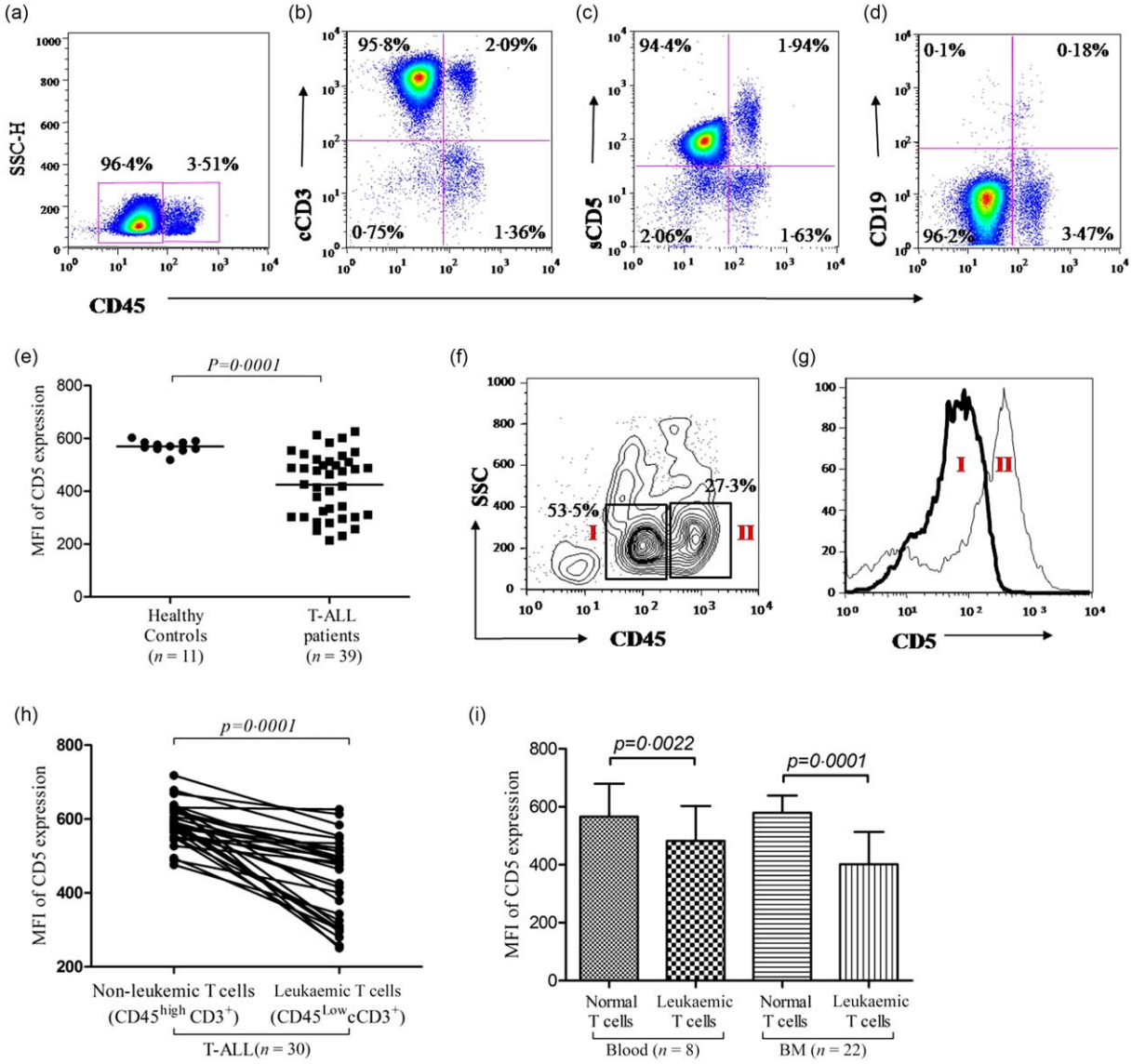

Fig. 1. Surface expression of CD5 on leukaemic cells from T cell acute lymphoblastic leukaemia (T-ALL) patients. (a) The flow cytometry plot shows gating of leukaemic ($CD45^{low}$) as well as non-leukaemic ($CD45^{high}$) cells. Cells are first gated on the mononuclear cells and then analysed on the basis of their CD45 expression and scatter profile. (b–d) Staining of mononuclear cells (MNCs) derived from T-ALL patients using specific monoclonal antibodies to various lineage markers (cCD3, CD5, CD19, etc.) defines the phenotype of T-ALL cases. Here, the expression of these markers is shown on the *y*-axis against CD45 (*x*-axis) in order to compare their expression on leukaemic ($CD45^{low}$) as well as non-leukaemic ($CD45^{high}$) cells. (e) Expression of CD5 as median fluorescence intensity (MFI) on T cells from healthy controls (HCs) ($n = 11$) and leukaemic T cells ($CD45^{low}SSC^{low}$) derived from T-ALL patients ($n = 39$) are shown ($P = 0{\cdot}0001$, Mann–Whitney *U*-test). (f,g) Leukaemic ($CD45^{low}$) and non-leukaemic ($CD45^{high}$) cells in the gates demarcated in E (I and II) were analysed for expression of CD5 (cell surface marker). It shows the gating strategy of a representative case. (h) Paired data of CD5 expression on leukaemic ($CD45^{low}$) and non-leukaemic cells ($CD45^{high}$) of patients ($n = 30$) are shown ($P = 0{\cdot}0001$, paired *t*-test). (i) Expression of CD5 as MFI on leukaemic and non-leukaemic (normal) T cells in samples [blood ($n = 8$) and bone marrow (BM, $n = 22$)] from T-ALL patients ($n = 30$) are shown. Significant differences are indicated with *P*-values in graphs. [Colour figure can be viewed at wileyonlinelibrary.com]

555450), CD34 (clone: 581, cat. no. 555822, all BD Pharmingen); peridinin-chlorophyll (PerCP)/cyanin 5-PE (Cy5PE)-conjugated CD45 (clone: HI30, cat. no. 555484), CD19 (clone: HIB19, cat. no. 555414) and CD5 (clone: UCHT2, cat. no. 555354; BD Pharmingen).

Isolation of cells from peripheral blood and bone marrow aspirate (BMA)

Briefly, mononuclear cells (MNCs) were isolated from heparinized blood and BMA by Ficoll Hypaque gradient centrifugation. The cell viability was checked by

Table 1. Demographic features of patients of acute T cell lymphoblastic leukaemia patients

Acute T cell lymphoblastic leukaemia patients	No. 39
Demographic characteristics	
Age (mean ± s.d.)	23·27 ± 14·57
Sex (male/female)	30/9
Diagnosis	
Percentage blast in blood smear	61·62 ± 26·52
Percentage blast in blood bone marrow	87·21 ± 22·02
WBC count	74 400 ± 84 895
Performance score (PS)	
PS 1	13
PS 2	12
PS 3	9
PS 4	5
Healthy controls (non-family members)	No. 11
Demographic characteristics	
Age (mean ± s.d.)	28·84 ± 6·44
Sex (male/female)	8/3

WBC = white blood cell; s.d. = standard deviation.

trypan blue dye exclusion test and was greater than 98% [22].

Surface and intracellular staining of cells

Isolated cells were incubated with labelled monoclonal antibodies in staining buffer [phosphate-buffered saline (PBS) + bovine serum albumin (BSA) + azide] for 15 min on ice. After washing twice with PBS, cells were fixed in 2% formalin, then resuspended in staining buffer and transferred in a FACS tube (BD Falcon, Bedford, MA, USA) for data acquisition within 24 h. For staining of intracytoplasmic antigens (e.g. CD3, IL-2), cells were permeabilized using permeabilization buffer (0·3% saponin; 84510; Fluka, Sigma-Aldrich) after fixation for 15 min at room temperature [22].

To measure the expression of sCD5 accurately in healthy as well as leukaemic T cells, the MNCs were first stained with fluorochrome-conjugated α-CD5 antibody (Fig. 3a,b; ii). Their level of expression [median fluorescence intensity (MFI)] was subtracted from that of cells first incubated with non-conjugated α-CD5 antibody and then incubated with fluorochrome-conjugated α-CD5 antibody (background fluorescence; Fig. 3a,b; iii). Similarly, the levels of expression of cCD5 were estimated by subtracting the MFI expression of permeabilized cells incubated with fluorochrome-conjugated α-CD5 antibody from the same cells, incubated first with non-conjugated α-CD5 antibody and then with fluorochrome-conjugated α-CD5 antibody (background fluorescence; Fig. 3a,b; v,vi, respectively). It should be noted that while measuring the levels of expression of cCD5, the surface molecules of CD5 (sCD5) were blocked before permeabilization to avoid their unwarranted contribution in the estimation of cCD5 (Supporting information, Fig. S2).

Flowcytometry and data analysis

Stained samples were acquired in a three-colour flow cytometer (FACSCalibur; Beckton Dickinson). The samples were kept at 4°C in the dark until acquisition was performed within 24 h of sample preparation [17]. Acquired data were analysed on FlowJo version 10.1.1. software (Tree Star, Inc., Ashland, OR, USA). During data analysis, all the gates were applied using fluorescence minus one (FMO) control. First, in a CD45 *versus* SSC plot, $CD45^{low}$ and $CD45^{high}$ cells were gated to distinguish the leukaemic and non-leukaemic cells, respectively. Hereafter, these gated cells were analysed for expression of lineage-specific markers (cCD3, CD5, CD19, CD10, CD13, CD33, MPO, etc.) to identify the type of leukaemic cells. Once confirmed with the diagnosis of T-ALL, the residual samples were subjected to functional assays.

Culture of mononuclear cells

In culture-based studies, cells were cultured (2×10^6 cells/ml) in 96-well microculture plates (U-bottomed plates; BD Falcon) in the presence of phorbol myristate acetate (PMA) (5 ng/ml, P8139; Sigma-Aldrich) and ionomycin (1 μm, Sigma-Aldrich) for 72 h at 5% CO_2 and 37°C. For cytokine detection assay, cells were incubated with stimulant for 24 h and monensin (Golgi transport inhibitor, 1 μM; Sigma Aldrich) was added in the last 6 h [22]. In blocking studies, unconjugated anti-CD5 monoclonal antibody (cat. no. 555350; BD Pharmingen) was mixed with MNCs (2×10^6/ml) prior to the addition of a stimulant.

Amplification of *CD5* gene-specific mRNA by reverse transcriptase–polymerase chain reaction (RT–PCR)

Organization of the *CD5* gene is shown in Fig. 2a. Total mRNA was extracted from the MNCs obtained from peripheral blood and bone marrow using Trizol reagent (Sigma-Aldrich). mRNA was converted into cDNA by RT–PCR. Quality was assessed using the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Isolated, precipitated and quantified cDNA was then utilized for the amplification of E1A and E1B transcripts of CD5. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (housekeeping gene) was used as a positive control. The following sets of primers were used: CD5 E1A (AT = 60·8°C), forward 5′-GATGCATGGCCTTGTCCTGTG-3′, reverse 5′-ACCGCA GGTGAGGGTGTCTGG-3′; CD5 E1B (AT = 58·1°C), forward 5′-TTGGTGTCTGAGGGGTTTTGT-3′, reverse 5′-TTCAGCCACTGCGTTGATCCT-3′; and GAPDH (AT = 58°C), forward 5′-AAAATCAAGTGGGGCGATGC-3′, reverse 5′-TGAGCTTGACAAAGTGGTCG-3′ [22].

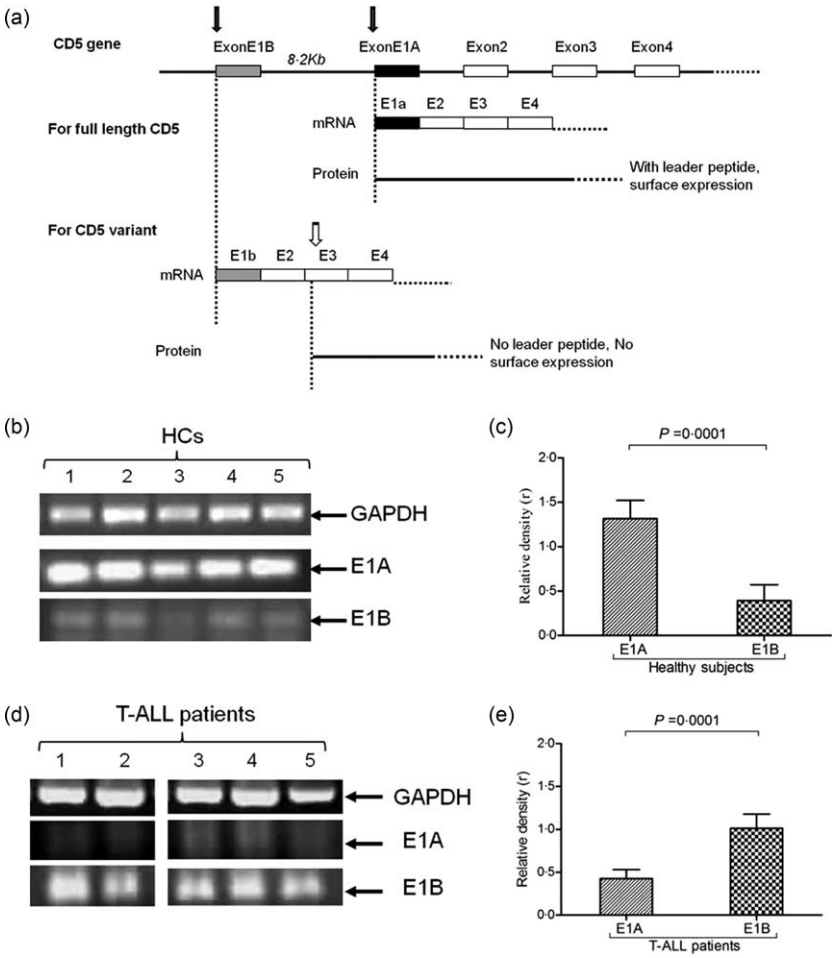

Fig. 2. Expression of early region 1 E1 A and E1B transcripts of CD5 in acute T cell lymphoblastic leukaemia (T-ALL). (a) The schematic diagram shows organization of an exon cluster of CD5. The diagram shows exon E1A and non-conventional exon E1B. Gel photograph and relative density (r) plot of semi-quantitative reverse transcriptase–polymerase chain reaction shows expression of exon E1A containing mRNA in (b,c) healthy controls (HCs, $n = 10$) and (d,e) T-ALL patients ($n = 12$). Expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene. Significance is shown as the *P*-value ($< 0{\cdot}05$) using the paired *t*-test. Data of five representative cases are shown.

Statistical analysis

Normal continuous variables are presented in mean ± s.d. However, non-normal covariants are shown in the median and interquartile range. The comparison of all continuous variables between the studied group, *t*-tests and Mann–Whitney tests were used. *P*-values less than 5% level of significance were regarded as significant. Analysis was performed using SPSS version 15.0. Graphs and figures were made in GraphPad Prism version 5.

Results

Reduced surface expression of CD5 on gated T cell blasts ($CD45^{low}$) among T-ALL patients compared to healthy controls

We evaluated the cell surface expression of CD5 on gated normal as well as T lymphoblasts from patients with T-ALL using flow cytometry-based phenotyping (Fig. 1a–d). Leukaemic blasts ($CD45^{low}$) of T-ALL patients ($n = 39$) showed significantly decreased levels of sCD5 (MFI) compared to T cells of healthy control subjects (HCs; $n = 11$, Fig. 1e; $P = 0{\cdot}0001$, Mann–Whitney test). Of 39 T-ALL cases, we chose 30 T-ALL cases where the non-leukaemic T cells were significantly present (no. of blood and BM samples were eight and 22, respectively). Levels of CD5 expression were observed on leukaemic ($CD45^{low}$) and non-leukaemic cells ($CD45^{high}$) of the same T-ALL cases (Fig. 1f,g). Paired data of CD5 expression on leukaemic and non-leukaemic cells show a significant down-regulation of expression (Fig. 1h; $P = 0{\cdot}0001$, paired *t*-test). To understand further the extent of down-regulation between the blood and BM samples of T-ALL cases, we analysed separately the MFI of CD5 expression on normal and leukaemic T cells. Our finding indicates down-regulation of CD5 expression, irrespective of the sample types (blood versus BM, Fig. 1i). To quantify the down-regulation of CD5 molecule on the surface of leukaemic T cells derived from T-ALL patients, molecules of equivalent soluble fluorophores (MESF) of sCD5 on the blasts and non-leukaemic T cells derived from T-ALL patients were calculated in terms of quantum MESF value from the standard curve. Significant reduction in the quantum MESF value in leukaemic T cells compared with

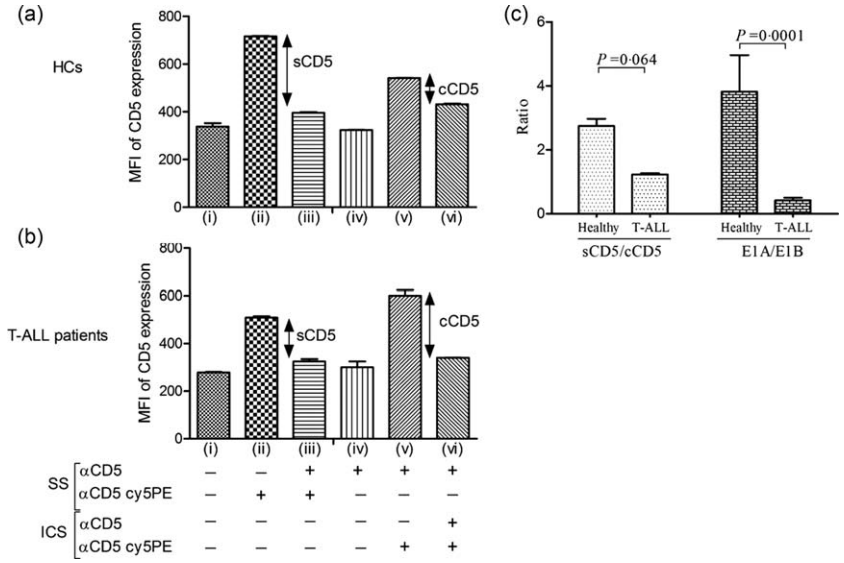

Fig. 3. Level of cytoplasmic CD5 (cCD5) among acute T cell lymphoblastic leukaemia patients. Using unconjugated and cyanin 5-phycoerythrin (Cy5PE)-conjugated anti (α)-CD5 antibody (of the same clone), we aimed to negate the background fluorescence shift, which helped to calculate the correct levels of cCD5 expression. In the bar diagrams, difference in median fluorescence intensity (MFI) of CD5 expression of column (ii) minus (iii) and column (v) minus (vi) suggest the surface (s) and cytoplasmic (c) expression of CD5, respectively. Bar diagram shows levels of cCD5 in (a) T cells of healthy controls (HCs) and (b) leukaemic T cells derived from T-ALL patients (showed by double-headed arrow). Each staining was performed in duplicate. The data shown are representative of five cases of HCs and T-ALL. (c) Bar diagram depicts the ratio of expression (MFI) of sCD5/cCD5 protein ($P = 0{\cdot}064$, unpaired *t*-test) and relative density (r) of a gel band of exon early region 1 (E1)A/E1B ($P = 0{\cdot}0001$, unpaired *t*-test) among HCs as well as T-ALL patients.

non-leukaemic T cells was observed (Supporting information, Fig. S1). Importantly, we found an inverse relation between performance score and percentage of down-regulation in per-cell CD5 expression (MFI; normal *versus* blast T cells) ($r^2 = 0{\cdot}3427$, $P < 0{\cdot}0001$). However, we failed to find significant correlations between the blast percentage and percentage of down-regulation in per-cell CD5 expression (data not shown).

Differential transcription of CD5 exons E1A and E1B regulates expression of CD5 among acute T cell lymphoblastic leukaemia patients

Expression of exons E1A and E1B has been linked to the surface and cytoplasmic expression of CD5 protein. However, this has not been investigated in T-ALL in the context of CD5 expression. Moreover, it would be interesting to explore whether this exonal switching is involved in down-regulation of CD5 on leukaemic T cells. To investigate the role of transcriptional switch between exons E1A and E1B containing mRNA in down-regulation the sCD5 on T-ALL cells, we measured their expression semi-quantitatively among cells derived from HCs and T-ALL patients. For this purpose, PBMCs from T-ALL patients with more than 98% blasts were selected. Exon E1B containing mRNA was predominant in the leukaemic T cells of T-ALL patients in comparison with that of magnetically sorted T cells derived from HCs (Fig. 2b,d). In contrast, exon E1A-containing mRNA were barely detectable in leukaemic T cells of T-ALL patients relative to the T cells derived from HCs (Fig. 2b,d). Densitometry analysis reveals the definitive shift of the ration between E1A/E1B towards the latter in the T-ALL blasts (Fig. 2c,e). To summarize, the data indicate that T cells derived from HCs express more exon E1A containing mRNA and fewer exon E1B containing mRNA (Fig. 2b). Conversely, cells of T-ALL origin express more exon E1B and fewer exon E1A containing mRNA (Fig. 2e), suggestive of more cCD5 and less sCD5 expression.

Accumulation of cytoplasmic CD5 (cCD5) in blasts of acute T cell lymphoblastic leukaemia patients

To demonstrate the levels and ratios of sCD5 and cCD5 in blasts of T-ALL patients, we stained cells derived from HCs and T-ALL patients with different combinations of conjugated and unconjugated (cold) anti-CD5 monoclonal antibodies (mAbs) of the same clone at the surface as well as intracellular levels (Fig. 3a,b; i–vi). Unconjugated antibodies were used to block the CD5 epitope either at the surface or at intracellular level to negate the background staining of either sCD5 or cCD5 accurately. Staining steps as well as saturating doses of antibodies were first titrated (data not shown). The differences in MFI of CD5 expression of the combination (ii) minus (iii) and combination (v) minus (vi) were taken as the accurate expression of sCD5 and cCD5, respectively.

Our results suggest the predominant expression of sCD5 on normal T cells derived from healthy subjects

with minimal cCD5. Conversely, leukaemic T cells of T-ALL patients express more cCD5 with fewer sCD5 relative to that of T cells of HCs (Fig. 3a,b). Furthermore, the ratio of sCD5 and cCD5 among T-ALL patients (sCD5↓ and cCD5↑) showed a definite decline when compared with that of HCs (sCD5↑ and cCD5↓, Fig. 3c). These findings are in concordance with the data of altered ratio of exons E1A and E1B transcripts, where leukaemic T cell-derived T-ALL patients express more exon E1B and fewer exon E1A.

Blocking of CD5 increases the capacity of non-leukaemic T cells to produce IL-2 among acute T cell lymphoblastic leukaemia patients

In order to determine the effect of CD5 blocking on the survival of leukaemic and non-leukaemic populations at various time-points (6, 12 and 48 h), we stimulated cells from T-ALL patients with PMA + ionomycin and measured the frequency of leukaemic and non-leukaemic populations. Upon treatment with PMA, neither leukaemic ($CD45^{low}cCD3^{+}$) nor non-leukaemic ($CD45^{high}cCD3^{+}$) T cells showed any change in their proportional frequency at 6, 12 and 48 h (Fig. 4a,b; Supporting information, Fig. S3). When CD5 molecules were blocked simultaneously, their frequency remained unaltered at 6 and 12 h. However, there was a significant increase in the frequency of non-leukaemic T cells ($P = 0{\cdot}049$) and a decrease in the frequency of leukaemic T cells ($P = 0{\cdot}040$) at 48 h of CD5 blocking (Fig. 4b). Intrigued by this observation, we measured the frequency of IL-2-producing leukaemic and non-leukaemic T cells in similar experiments (Supporting information, Fig. S4). Neither the stimulation of cells with PMA alone nor PMA with CD5 blocking showed any significant change in the frequency of IL-2-producing leukaemic T cells from T-ALL patients (Fig. 4c). Conversely, the non-leukaemic T cells from T-ALL patients showed a significant increase in the frequency of IL-2-producing T cells from 6 to 48 h (Fig. 4d; 6 and 12 h, $P = 0{\cdot}016$; 6 and 48 h, $P = 0{\cdot}023$). This was accompanied by an increase in the proportion of normal T cells at the 48-h time-point. Conversely, we observed a concomitant reduction in the proportion of T cell blasts in the culture over time. To conclude further, the event count (per 1 00 000 acquired events) indicates a similar observation (Supporting information, Fig. S5). Interested by this observation, we tried to determine that either the proliferation of non-leukaemic T cells and/or reduction in the proliferation of leukaemic cells contribute towards the proportional increase in the frequency of unaffected non-leukaemic T cells. Our results show a decrease in the frequency of proliferating leukaemic T cells upon blocking with anti-CD5 antibody after 12 h of PHA stimulation. It was interesting to note that the unaffected non-leukaemic T cells showed no increase in their frequency under similar conditions, at least at 12 h of stimulation. It should also be noted that we did not observe any cell death at 12 h of stimulation.

Discussion

CD5 is expressed on T cells and a minor subset of B cells, which are identified as self-replicating B1 cells, present in celoemic body cavities [5,6]. Signalling through CD5 inhibits the TCR–CD3-mediated activation of T cells [7]. Many of these effects, i.e. proliferation, IL-2 production, are mediated predominantly by the recruitment of Src-homology 2-domain-containing phosphatase (SHP-1) to the cytoplasmic tail of CD5 [8]. CD5 is thus considered as a negative regulator of TCR-mediated activation [8,21]. However, the blasts from a sizable fraction of T-ALL patients fail to show surface CD5 expression and are therefore diagnosed as $cCD3^{+}CD7^{+}CD5^{-}$. Considering its role, we intended to investigate the levels of CD5 expression on the leukaemic blasts of T ALL patients and what implications it would have on the biology of T ALL. We observed a definitive reduction in the cell surface levels of CD5 on the blasts. However, the autologous normal T cell compartment showed higher levels of sCD5 (Fig. 1h). Down-regulation of CD5 on $CD8^{+}$ T cells are already shown in familial haemophagocytic lymphohistiocytosis with perforin gene mutations and in Epstein–Barr Virus (EBV)-infected $CD8^{+}$ T lymphocytes [23,24]. Similarly, the B cells from patients with systemic lupus erythematosus (SLE) show reduced expression levels of membrane CD5 [25]. In immunophenotyping-based diagnostic services, we provide sCD5 positivity based on their isotype control and/or FMO control. It should be noted that cases that showed sCD5 positivity in immunophenotyping-based diagnostic services were actually showing down-regulation of sCD5 in comparison to their non-leukaemic T cell compartment.

Renaudineau *et al.* [14] showed that exonal switching between alternative forms of exon 1 of the *CD5* gene regulates CD5 expression in human B lymphocytes. To evaluate the possible role of such exonal switching in the observed CD5 down-regulation, we measured the ratio of E1A and E1B in both the leukaemic blast in T-ALL patients and T cells from healthy subjects. The reciprocal dominance of E1A and E1B in normal T cells and blast T cells of T-ALL patients, respectively (Fig. 2b–e), strongly suggests exonal switching from the former to the latter (E1A to E1B) during leukaemogenesis. Our data demonstrate further that exonal switching correlates closely with the cell surface expression of CD5 molecules on T cells (Fig. 3c). We observed a striking correlation between the ratios of sCD5/cCD5 and E1A/E1B. We concluded that surface *versus* intracellular levels of CD5 are regulated by E1A to E1B exonal switching, and this is linked to down-regulation of the sCD5 expression in the leukaemic T cells of T-ALL patients (Fig. 5). This also indicates that this is possibly associated

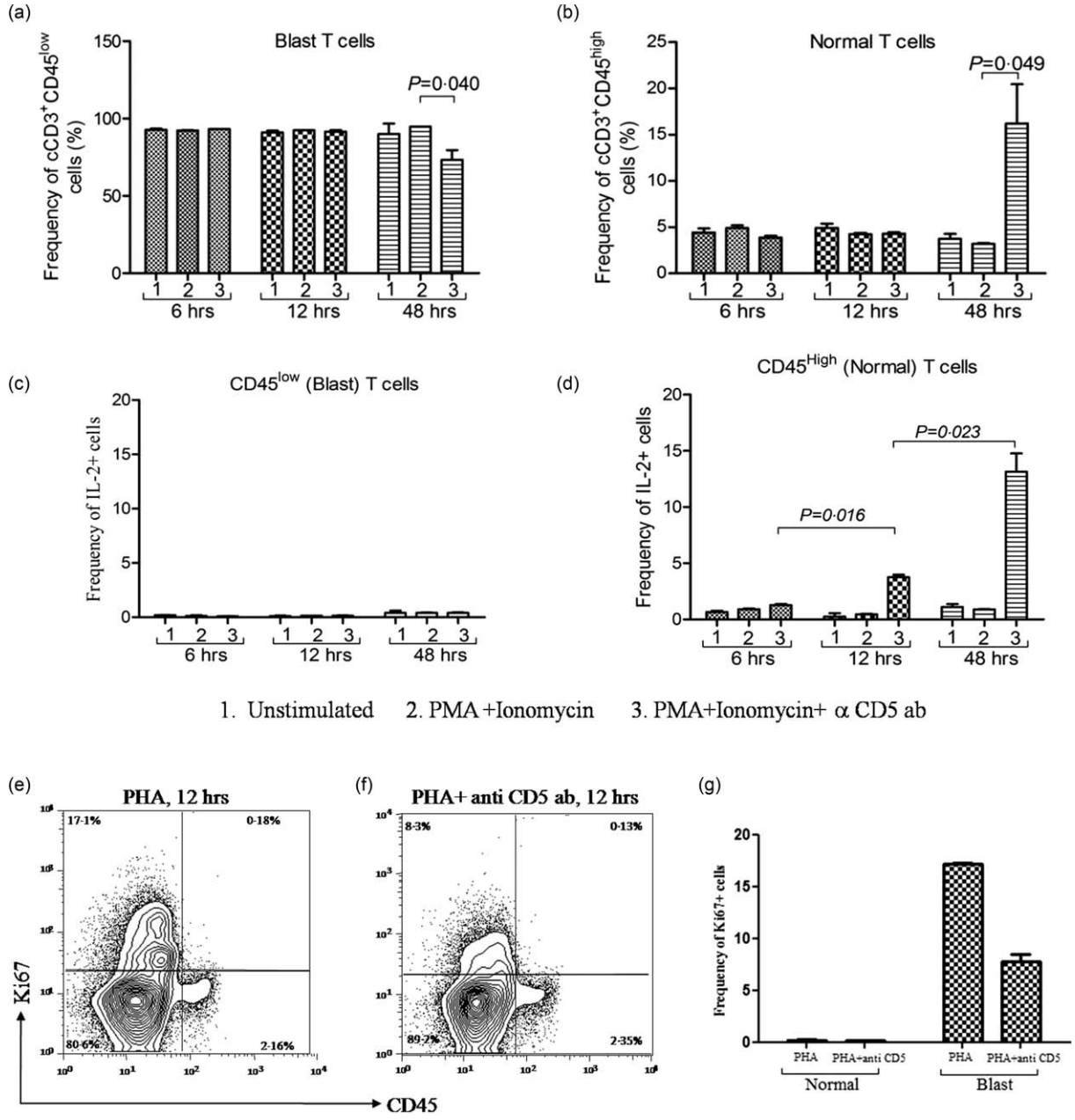

Fig. 4. Effect of CD5 inhibition on survival of non-leukaemic T cells from acute T cell lymphoblastic leukaemia patients (T-ALL). Cells from T-ALL patients cultured under different conditions [1: untreated, 2: polyclonal stimulation phorbol–myristate–acetate (PMA) + ionomycin and 3: polyclonal stimulation (PMA+ ionomycin) along with anti-CD5 antibodies] at 6, 12 and 48 h were analysed, respectively (Supporting information, Fig. S3). (a,b) Frequency of non-leukaemic T ($CD45^{high}cCD3^{+}$) and leukaemic T cell ($CD45^{low}cCD3^{+}$) cells from T-ALL patients in the gates demarcated in (a) were analysed and shown in the representative bar diagram from four independent experiments. (c,d) Interleukin (IL)-2 productions of non-leukaemic and leukaemic T cells were analysed under different conditions (untreated, PMA and PMA + anti-CD5 antibody) at 6, 12 and 48 h and shown in the representative bar diagram from four independent experiments. Representative flow cytometry plots are shown in Supporting information, Fig. S4. Significant differences are indicated with *P*-values in graphs. (e–g) Proliferation of leukaemic ($CD45^{low}$) and non-leukaemic ($CD45^{high}$) cells, as measured by Ki67 positivity, is shown after 12 h of PHA stimulation upon blocking with anti-CD5 antibody. The cumulative plot shows a decrease in the proliferation of leukaemic cells ($Ki67^{+}$ in $CD45^{low}$).

with leukaemic transformation of T cells, as normal T cells of T-ALL patients behave in an exactly opposite manner in terms of CD5 surface expression or intracellular accumulation. However, the mechanism underneath this exonal switch has not yet been elucidated. Garaud *et al.* suggested that the promoter region of the CD5–E1B isoform is demethylated in B cells from patients with SLE, but not in healthy controls [25]. Therefore, the status of methylation

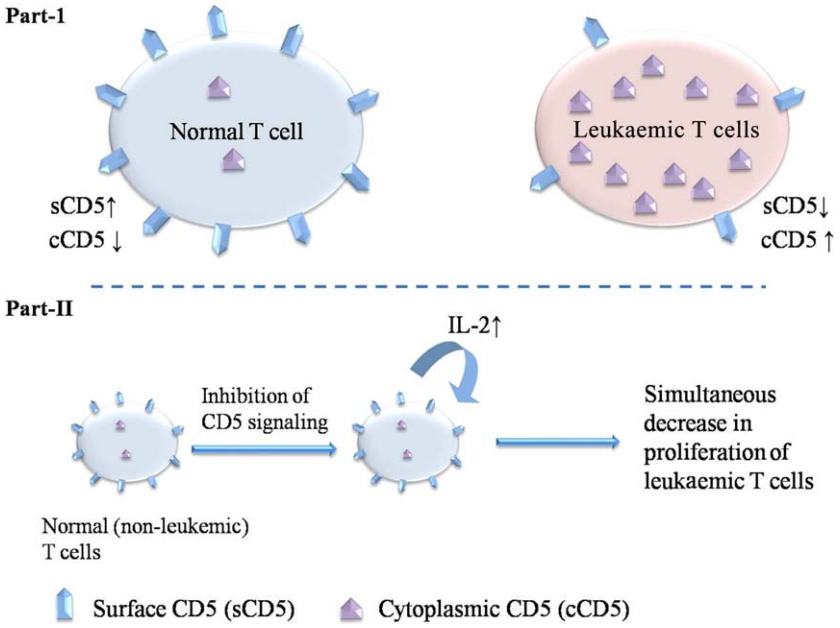

Fig. 5. In the first part of this schematic diagram, the predominant expression of early region 1 (E1)A and E1B in normal T cells and blasts T cells of acute T cell lymphoblastic leukaemia patients (T-ALL), respectively, strongly suggests exonal switching from the former to the latter (E1A→E1B) during leukaemogenesis. Our data demonstrate further that exonal switching correlates closely with the cell surface as well as intracellular expression of CD5 molecules on T cells. In the second part, the non-leukaemic T cells from T-ALL patients showed a significant increase in the frequency of interleukin (IL)-2-producing T cells upon blocking the CD5. It also shows a simultaneous decrease in the frequency of proliferating leukaemic T cells upon blocking with anti-CD5 antibody after 12 h of stimulation. [Colour figure can be viewed at wileyonlinelibrary.com]

in the promoter region of CD5E1A and E1B isoforms should be investigated.

Suppression of the immune surveillance by normal T cells among leukaemic patients, including T-ALL, is well known. Cancer cells may suppress host immunity by diverse mechanisms such as contact-dependent inhibition, inhibition of IL-2 receptor expression and down-regulation of HLA proteins [26,27]. Here, we show that the down-regulation of CD5 is associated closely with the leukaemic transformation of T cells. Furthermore, we evaluated the status of the host immune surveillance by residual normal T cells, which express a higher level of CD5 on their surface compared to autologous leukaemic T cells. To understand this, we blocked the CD5 expressed on the T cells (primarily normal T cells) from T-ALL patients. This resulted in a significant decrease in blast proliferation, with a simultaneous increase in the IL-2-producing non-leukaemic normal T cells (Fig. 5). Interestingly, polyclonal activation of T cells along with CD5 blocking induced expansion of normal T cells which otherwise failed to proliferate. These T cells could produce IL-2 only when CD5 signalling was inhibited. Our results indicate the critical role of CD5 in the pathobiology of T cell leukaemia. First, as an antagonist of TCR-mediated signalling, CD5 inhibits several T cell functions, such as proliferation, IL-2 production, interferon (IFN)-γ production and activation. Therefore, a cell surface form of CD5 may act as a checkpoint of excessive T cell activation and proliferation. An exonal switch in the transcription of CD5 gene may be involved in the loss of cell surface CD5, with its intracellular accumulation resulting in the removal of negative regulatory signalling. This may contribute to the uncontrolled growth and activation of T cells, leading to neoplastic transformation. Besides this, the exonal switching (E1A→E1B) may also regulate the functional behaviour of the T cells. It has already been shown that the exonal switch is linked to cytokine production by the T cells [25,28]. Therefore, it would be important to understand the role of exonal switch (E1A→E1B) in the cytokine(s) production of T cells upon activation.

Performance score (PS) is an index of the patient's ability to perform certain routine activities that may be considered as a reflection of disease severity. Our finding indicates clearly an inverse correlation between PS and the percentage of down-regulation in per-cell CD5 expression (MFI, normal T cells *versus* leukaemic T cells) ($r^2 = 0{\cdot}3427$, $P < 0{\cdot}0001$). Most importantly, our study demonstrates that inhibiting CD5 signalling may expand the normal IL-2-producing T cells proportionally, which are known to be suppressed in leukaemias, and their generation may be associated with a reduction of proliferating T cell blasts. This is suggestive of the fact that inhibiting CD5 signalling may rescue the host T cells, which may restore the host immune response and anti-leukaemic effect. It will be interesting to define the phenotype and function of anti-leukaemic T cells expanded by inhibition of CD5 signalling. This may have a significant impact upon the immunotherapy of T cell leukaemias.

Acknowledgements

All experiments were carried out in the same laboratory. The authors thank all the patients and control subjects who volunteered participation in this study. The authors acknowledge the support from the late Dr Arundhati Panigrahi, who provided Quantum MESF beads for our experiments. A part of the study was supported by the grant from The Department of Biotechnology, Government of India (GoI). The authors acknowledge the support received at the

flow cytometry data analysis facility, Centre for Medical Diagnostic and Research (CMDR), MNNIT Allahabad for analysis of data. We are also thankful to Dr J. Philip McCoy, Senior Scientist. Flow Cytometry Core, National Heart, Lung and Blood Institute (NHLBI), National Institute of Health (NIH), Bethesda, MD, USA for his valuable suggestions and editing. The study was carried out as part of routine diagnostic services based on flow cytometer based immunophenotyping at the Cellular Immunology Laboratory, Department of Transplant Immunology and Immunogenetics, All India Institute of Medical Sciences (AIIMS), New Delhi. The authors acknowledge the support received from AIIMS, New Delhi and Department of Biotechnology, Government of India (Ref. no. 6242-P101/RGCB/PMD/DBT/AMKR/2015).

Disclosure

None of the authors have any competing interests.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Quantification of CD5 down-regulation by quantum molecules of equivalent soluble fluorophores (MESF) beads: (a) five beads (of the same size, including

unstained beads) having different fluorescence in the fluorescein isothiocyanate (FITC) channel (FL-1) were mixed and acquired by flow cytometer. The bivariant fluorescence activated cell sorter (FACS) plot shows the varying fluorescence by bead (tagged with FITC). (b) Overlaid histogram of all five beads (including unstained beads) and unstained bead (filled histogram) show the gating strategies of peak of different bead. The median fluorescence intensity (MFI) of each peak was calculated. (c) Overlaid histogram of bead-FITC (all five beads), unstained cells (light filled) and stained (for CD5-FITC) non-leukaemic cells (dark filled; $CD45^{high}$) showed expression of CD5. Their MFI was calculated using FlowJo software. (D) Similarly, leukaemic cells (dark filled; $CD45^{low}$) showed decreased expression of CD5. Their MFI was also calculated. (e) A linear curve was plotted between MESF value (on the *x*-axis) and MFI of each bead (peak). Using linear curve, MESF values were quantified for the expression of CD5 on non-leukaemic and leukaemic cells derived from acute T cell lymphoblastic leukaemia (T-ALL) patients, suggesting down-regulation of CD5 expression ($P = 0{\cdot}057$, paired *t*-test) on leukaemic cells. (f) Bar diagram showed surface expression of CD5 (using quantum MESF FITC beads) on non-leukaemic and leukaemic T cells of T-ALL patients ($n = 3$).

Fig. S2. Schematic representation shows strategies used to accurately measure the expression of surface CD5 (sCD5) and cytoplasmic CD5 (cCD5) in T cells. The mononuclear cells (MNCs) were first stained with fluorochrome conjugated α-CD5 antibody (condition ii). Their level of expression [median fluorescence intensity (MFI)] was subtracted from the same cells, incubated first with non-conjugated α-CD5 antibody and then with fluorochrome-conjugated α-CD5 antibody (corrected background fluorescence; condition iii). Similarly, the level of expression of cCD5 was estimated by subtracting the MFI expression of permeabilized cells incubated with fluorochrome-conjugated α-CD5 antibody (condition v) from the same of cells first incubated with non-conjugated α-CD5 antibody and then incubated with fluorochrome-conjugated α-CD5 antibody (corrected background fluorescence for intracellular expression; condition vi). This must be noted that while measuring the levels of expression of cCD5, the surface molecules of CD5 (sCD5) were blocked before permeabilization to avoid their unwarranted contribution in the estimation of cCD5.

Fig. S3. (A) Flow cytometry plot shows non-leukaemic T ($CD45^{high}cCD3^{+}$) and leukaemic T cells ($CD45^{low}cCD3^{+}$) from acute T cell lymphoblastic leukaemia (T-ALL) patients under different conditions [column 1: untreated, column 2: phorbol–myristate–acetate (PMA) and column 3: PMA+anti-CD5 antibody] at 6, 24 and 48 h, respectively.

Fig. S4. (a) Flow cytometry plot showed production of interleukin (IL)-2 by non-leukaemic T ($CD45^{high}cCD3^{+}$) and leukaemic T ($CD45^{low}cCD3^{+}$) cells from acute T cell lymphoblastic leukaemia (T-ALL) patients under different conditions [column 1: untreated, column 2: phorbol–myristate–acetate (PMA) and column 3: PMA+anti-CD5 antibody] at 6, 24 and 48 h, respectively.

Fig. S5. Cells from acute T cell lymphoblastic leukaemia (T-ALL) patients cultured under different conditions [1: untreated, 2: polyclonal stimulation, phorbol–myristate–acetate (PMA) + ionomycin and 3: polyclonal stimulation, PMA+ ionomycin along with anti-CD5 antibody] at 48 h were analysed, respectively. The event count in gated normal unaffected T cells ($CD45^{high}$) and leukaemic T cells ($CD45^{low}$) (1×10^5 acquired events) are shown.