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ARTICLE

Acute lymphoblastic leukemia

Leukemia-induced dysfunctional TIM-3^{+}CD4^{+} bone marrow T cells increase risk of relapse in pediatric B-precursor ALL patients

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Abstract

Interaction of malignancies with tissue-specific immune cells has gained interest for prognosis and intervention of emerging immunotherapies. We analyzed bone marrow T cells (bmT) as tumor-infiltrating lymphocytes in pediatric precursor-B cell acute lymphoblastic leukemia (ALL). Based on data from 100 patients, we show that ALL is associated with late-stage CD4^{+} phenotype and loss of early CD8^{+} T cells. The inhibitory exhaustion marker TIM-3 on CD4^{+} bmT increased relapse risk (RFS = 94.6/70.3%) confirmed by multivariate analysis. The hazard ratio of TIM-3 expression nearly reached the hazard ratio of MRD (7.1 vs. 8.0) indicating that patients with a high frequency of TIM-3^{+}CD4^{+} bone marrow T cells at initial diagnosis have a 7.1-fold increased risk to develop ALL relapse. Comparison of wild type primary T cells to CRISPR/Cas9-mediated TIM-3 knockout and TIM-3 overexpression confirmed the negative effect of TIM-3 on T cell responses against ALL. TIM-3^{+}CD4^{+} bmT are increased in ALL overexpressing CD200, that leads to dysfunctional antileukemic T cell responses. In conclusion, TIM-3-mediated interaction between bmT and leukemia cells is shown as a strong risk factor for relapse in pediatric B-lineage ALL. CD200/TIM-3-signaling, rather than PD-1/PD-L1, is uncovered as a mechanism of T cell dysfunction in ALL with major implication for future immunotherapies.

Introduction

Acute lymphoblastic B-precursor leukemia (BCP-ALL) is the most common malignancy in childhood and adolescence. Relapse is the most important cause for treatment failure and occurs in about 10–20% of patients [1]. Known prognostic criteria for relapse prediction and risk stratification are genetic risk factors, white blood cell count (WBC), age, and minimal residual disease (MRD) after the end of induction therapy. In contrast to solid malignancies [2], T cell interaction with leukemic cells have not yet been investigated as prognostic factor. Since B-precursor ALL cells are antigen-presenting cells (APCs), we hypothesized an interaction between bone marrow T cells (bmT) and leukemic cells and defined bmT cells as tumor-infiltrating lymphocytes (TILs). T cells are known to interact with malignant cells through co-inhibitory and co-stimulatory molecules [3]. The PD-1/PD-L1 and CTLA-4/CD80/CD86 axes are well-known modulators of T cell responses against malignant cells and can be targeted successfully for immunotherapy of cancer [4, 5]. However, these checkpoint inhibitors have been less efficient in the treatment of ALL [6, 7], suggesting that different markers and mechanisms might be involved in the exhaustion of bmT cells and the escape from immunosurveillance of ALL. The role of co-stimulation, co-inhibition and T cell exhaustion in pediatric ALL remains largely with unresolved questions.

Supplementary information The online version of this article (https://doi.org/10.1038/s41375-020-0793-1) contains supplementary material, which is available to authorized users.

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T cell immunoglobulin and mucin-domain containing-3 (TIM-3) has been identified in 2002 as an inhibitory molecule expressed on $CD4^+$ and $CD8^+$ cells in response to proinflammatory signals [8], as well as on cells of the innate immune system. Expression of TIM-3 can be induced on T cells by inflammatory cytokines such as Interleukin (IL)-2, IL-7, IL-12, IL-15, IL-21, IL-27, and transforming growth factor beta TGF-β [9–12]. Galectin-9, high mobility group box 1 protein (HMGB1), phosphatidylserine (PtdSer), and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) were identified as binding partners of TIM-3 and mediate TIM-3 function [13–15]. Initial studies showed that TIM-3 is essential for induction of T cell tolerance in autoimmune disease and immune tolerance during pregnancy [8, 16, 17]. TIM-3-blocking antibodies led to hyper-acute T cell mediated experimental autoimmune encephalomyelitis and uncontrolled macrophage activation in mouse models [8]. In models of chronic viral infection, TIM-3 was found to be abundantly expressed on virus-specific $CD8^+$ T cells associated with T cell exhaustion and dysfunction [18, 19]. In malignant disease, TIM-3 expression was described on dysfunctional and exhausted tumor-specific and tumor-infiltrating lymphocytes (TILs) in various solid malignancies [10, 20–24]. In adult patients suffering from ALL the proportion of $PD1^+TIM3^+$ double-positive $CD4^+$ T cells differentiated a poor survival group [25]. Until recently, TIM-3 expression has not been evaluated in the bone marrow of patients suffering from pediatric acute lymphoblastic leukemia.

Here, we hypothesize a prognostic relevance of co-stimulation and co-inhibition between pediatric BCP-ALL blasts and bmT cells and address three questions: First, are there differences in the blast to bmT cell interaction between healthy and malignant cells? Second, can we identify markers with prognostic relevance and third, are described markers involved in T cell dysfunction/T cell exhaustion? We describe that TIM-3 on T cells is linked to decreased T cell responses in vitro and an increased relapse risk in vivo. Our work shows that ALL blast-mediated interaction with the co-inhibitory molecule CD200 increase expression of TIM-3 on T cells, leading to attenuated activation and proliferation of bmT cells. The TIM-3/CD200 interaction in pediatric B-precursor ALL can extend current risk stratification and immunotherapeutic treatment advances.

Methods

Patients

Patients' and healthy donors' characteristics are shown in Table 1. This study was approved by the Institutional Ethical

Table 1 Characteristics of cALL/pre-B ALL patients and healthy donors.

Characteristic	BCP-ALL patients ($n = 100$)	Healthy donors ($n = 13$)
Gender (male/female)	50/50	6/7
Age at diagnosis/sampling (mean, range)	6 (1–17)	7 (1–13)
<10 years (%)	81.0	76.9
≥10 years (%)	19.0	23.1
Relapse rate (%)	14.0	
Early relapse (%)	21.4	
Late relapse (%)	78.6	
Time until relapse (mean months, range)	29 (14–62)	
Time until last follow-up (mean months, range)	97.7 (2–139)[a]	
Relapse-free survival (%)	86.0	
Event-free survival (%)	84.0	
Mortality (%)	4.0	
MRD day 29		
N.a. (%)	0.0	
$<10^{-3}$ (%)	72.0	
$\geq 10^{-3}$ (%)	28.0	
MRD day 43		
N.a. (%)	12.0	
$<10^{-3}$ (%)	80.0	
$\geq 10^{-3}$ (%)	8.0	
Therapy response		
N.a. (%)	0.0	
CCR (%)	94.0	
LR (%)	6.0	
BCR-ABL translocation		
N.a. (%)	4.0	
Negative (%)	96.0	
Positive (%)	0.0	
MLL-AF4 translocation		
N.a. (%)	5.0	
Negative (%)	95.0	
Positive (%)	0.0	
TEL-AML1 translocation		
N.a. (%)	5.0	
Negative (%)	66.0	
Positive (%)	29.0	

Early relapse: <18 months after diagnosis; late relapse: ≥18 months after diagnosis; time until last follow-up: includes only patients without relapse. *MRD* minimal residual disease; *n.a.* data not available; *CCR* continuous complete remission (<5% leukemic blasts in bone marrow, regeneration, no extra-medullary site); *LR* late response (no remission on day 29, remission after 2nd HR1 block); days according to COALL-07-03 study protocol.

[a]Includes two deaths (two and seven months after initial diagnosis).

Review Board ("Ethikkommission bei der LMU München"), approval number 435–15/17–163, and the Institutional Ethical Review Board of the COALL trials 03–07 and 08–09, approval number 2077 and PVN3409. CoALL has been registered under www.clinicaltrials.gov: GPOH-COALL08–09 EU-21076/NCT01228331. The study was performed in accordance with the Declaration of Helsinki. Patients/healthy donors or their legal guardians have given written informed consent or analysis was performed from anonymized samples from the biobank. Bone marrow samples from cALL and pre-B ALL patients were kindly provided by the COALL study center and the Hauner Hematology Biobank. Bone marrow samples from healthy children without any evidence of malignancy or immunologic diseases, were used as an age-matched cohort (Table 1). A validation cohort of 40 bone marrow samples (BCP-ALL and T-ALL) was analyzed in order to validate the results of TIM-3^{+}CD4^{+} bone marrow T cells in a completely different cohort of patients (Supplementary Table 1).

Flow cytometry

Bone marrow cells of ALL patients and healthy donors were stained with CD10-BUV737, CD19-BUV496, CD27-BUV395, CD3-BUV395, CD4-BV650, CD40-BB515, CD62L-BB515, CTLA-4-APC (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA), 2B4-APC, CD45RO-PE, CD70-PerCP/Cy5.5, CD8-PerCP/Cy5.5, CD80-PE, CD86-BV650, HVEM-PE/Cy7, PD-1-BV421, PD-L1-BV421, TIM-3-BV785 (Biolegend, San Diego, California, USA), LAG-3-PE (R&D Systems, Minneapolis, Minnesota, USA), Fixable Viability Dye eFluor 780 (eBioscience/Thermo Fisher Scientific, Waltham, Massachusetts, USA). Intracellular stains were performed using Fix & Perm Cell Permeabilization Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's information. TIM-3 knockout and transduction rate was determined by flow-cytometric stain with TIM-3-BV421 (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). Activation of primary T cells was analyzed by flow-cytometric stain with CD69-PE-Vio 770 (Miltenyi Biotec, Bergisch Gladbach, Germany) and 4-1BB-BV421 (Biolegend, San Diego, California, USA). To measure TIM-3 induction by CD200, T cells were stained with 7-AAD Viability Staining Solution, TIM-3-BV421 (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA), CD3-PE-Vio770, CD4-VioGreen, and CD8-APC-Vio770 (Miltenyi Biotec, Bergisch Gladbach, Germany). Flow cytometric measurements were performed on a MACSQuant Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany) or BD LSRFortessa (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA).

Retroviral transduction

For retroviral transduction, human TIM-3 coding sequence (accession no. NM_032782) including a Kozak sequence was cloned into pMP71 (kindly provided by Christopher Baum, Medizinische Hochschule Hannover, Hannover, Germany) via EcoRI and NotI (pMP71_TIM-3). pMP71_TIM-3 and helper plasmids were transfected into 293T cells using TransIT-293 Transfection Reagent (Mirus Bio, Madison, Wisconsin, USA) according to the supplier's information. Retroviral supernatant was harvested 48 h later and used for transduction of primary T cells from healthy donors. Therefore, 24-well plates were coated with 2.5 µg RetroNectin Reagent (Takara Bio, Kusatsu, Japan) per well at 37 °C for 2 h. Plates were blocked with 2% Albumin Fraction V (Carl Roth, Karlsruhe, Germany) in PBS (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 30 min and washed with a 1:40 dilution of HEPES 1 M (Thermo Fisher Scientific, Waltham, Massachusetts, USA) in PBS. Virus supernatant was harvested and filtered (0.45 µm). One milliliter virus supernatant was transferred to each well of the plate and centrifuged $3000 \times g$ for 90 min at 32 °C. Virus supernatant was discarded and 1×10^6 T cells in 1 ml TexMACS GMP medium (Miltenyi Biotec, Bergisch Gladbach, Germany)/2.5% human AB serum (Institute for Clinical Transfusion Medicine, Ulm, Germany) +12.5 ng/ml human IL-7 and IL-15, premium grade (Miltenyi Biotec, Bergisch Gladbach, Germany) +2 µg/ml Protamine sulfate (Sigma-Aldrich, Taufkirchen, Germany) were added. Plates were centrifuged for 10 min at 450 g, 32 °C and washed 48 h after transduction. CD200 overexpression (accession no. NM_005944.6) in Nalm-6 cells was done in a comparable way (medium: RPMI-1640 (Biochrom, Berlin, Germany) +10% fetal calf serum (Sigma-Aldrich, Taufkirchen, Germany)).

TIM-3 induction assay

T cells were isolated from PBMCs of healthy donors using EasySep Human T Cell Enrichment Kit (STEMCELL Technologies, Vancouver, Canada) according to the manufacturer's information. T cells were activated with T Cell TransAct, human (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's information. T cells were cultured in TexMACS GMP medium (Miltenyi Biotec, Bergisch Gladbach, Germany)/ 2.5% human AB serum (Institute for Clinical Transfusion Medicine, Ulm, Germany) +12.5 ng/ml human IL-7 and IL-15, premium grade (Miltenyi Biotec, Bergisch Gladbach, Germany) and splitted every two to three days. After expansion for 12–14 days, T cells were used for TIM-3 induction assays. Ninety-six-well plates (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were coated with 1 µg/ml Recombinant Human CD200 Fc Chimera Protein (R&D Systems,

Minneapolis, Minnesota, USA) in PBS for 2 h at 37 °C. Mouse IgG1 Isotype Control-coated (R&D Systems, Minneapolis, Minnesota, USA) plates served as control. After 24 h, TIM-3 expression on $CD4^+$ T cells was analyzed by flow cytometry (geometric mean fluorescence intensity) and quantitative PCR was performed on a 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using Quick RNA Microprep Kit (Zymo Research, Irvine, California, USA) for RNA isolation, QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) for cDNA synthesis and Power SYBR Green PCR Master Mix for qPCR reaction (Thermo Fisher Scientific, Waltham, Massachusetts, USA). TIM-3 expression was normalized to T cells cultured on isotype control-coated plates. For qPCR, $CD4^+$ T cells were sorted using EasySep Human $CD4^+$ T Cell Isolation Kit (STEMCELL Technologies, Vancouver, Canada). Primer sequences for quantitative PCR: TCCCTTTGACTGTGTCCTGC (forward), AGTCCTGAGCACCACGTTG (reverse). To evaluate TIM-3 induction by CD200 expression on leukemic cell lines, Nalm-6 cells were transduced with human CD200 as described above. T cells were co-cultured with Nalm-6 wild type or CD200-transduced cells at a 1:1 ratio for 24–72 h. TIM-3 expression on $CD4^+$ T cells was analyzed by flow cytometry and qPCR as described above. TIM-3 expression was normalized to T cells co-cultured with Nalm-6 wild type.

CRISPR/Cas9-mediated TIM-3 knockout

TIM-3 gRNA was designed using crispr.mit.edu (PAM underlined): GTGGAATACAGAGCGGAGGTCGG. For CRISPR/Cas9-mediated TIM-3 KO, Alt-R CRISPR-Cas9 tracrRNA and Alt-R CRISPR-Cas9 crRNA (both from Integrated DNA Technologies, Coralville, Iowa, USA) were mixed 1:1 and heated 5 min at 95 °C. Alt-R S.p. Cas9 Nuclease 3NLS (Integrated DNA Technologies, Coralville, Iowa, USA) was mixed with the gRNA complex and Alt-R Cas9 Electroporation Enhancer (Integrated DNA Technologies, Coralville, Iowa, USA) and incubated 15 min at room temperature. For electroporation, buffer 1 M [26] was used on a Nucleofector 2b Device according to the manufacturer's instructions (Lonza, Basel, Switzerland). After electroporation, T cells were immediately transferred to fresh medium. For the comparison of knockout cells to wild type, wild type cells had to undergo the same procedures with a non-targeting gRNA (Alt-R CRISPR-Cas9 Negative Control crRNA #1; Integrated DNA Technologies, Coralville, Iowa, USA).

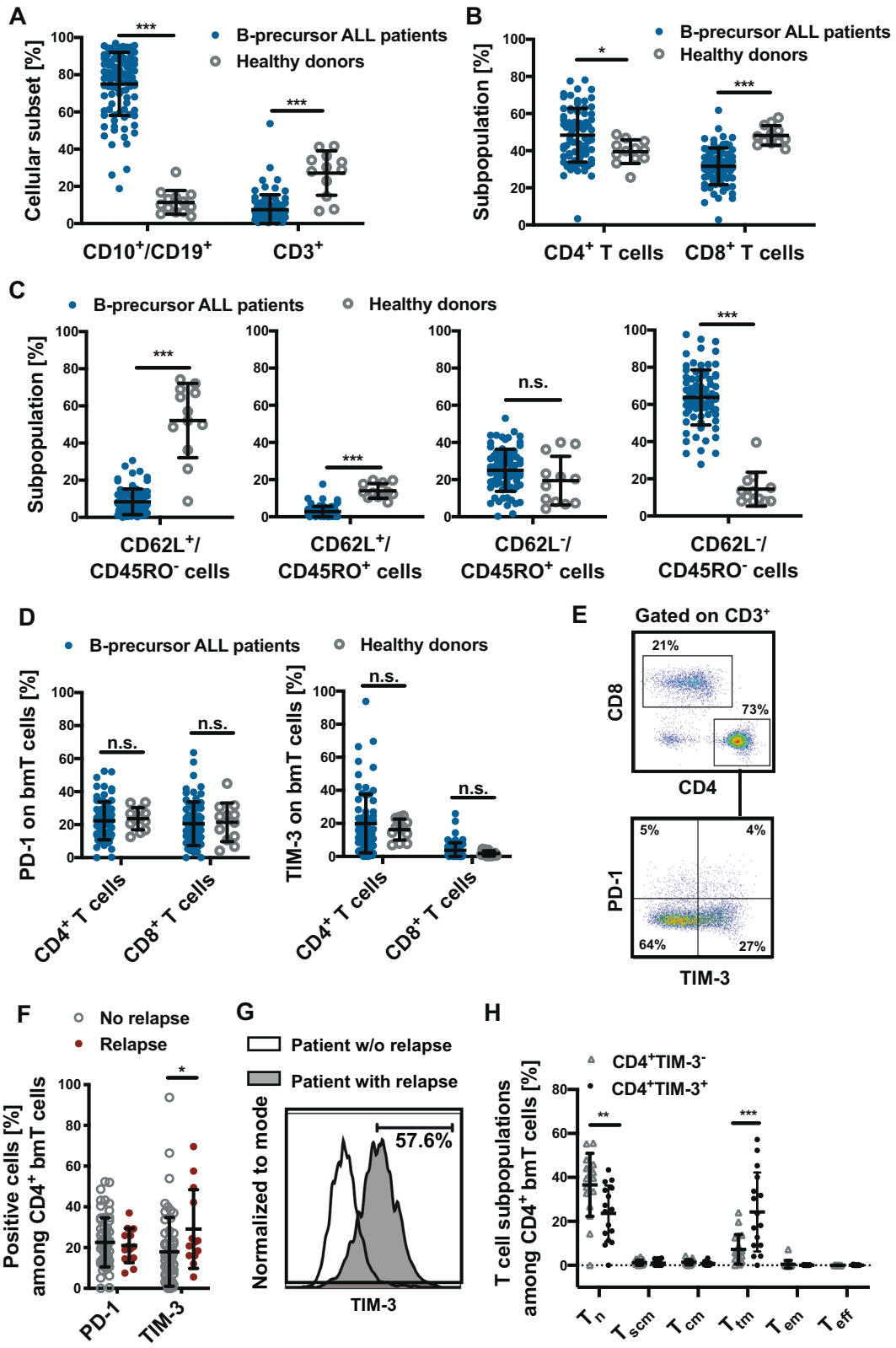
Statistics

Event-free survival (EFS) and relapse-free survival (RFS) was estimated using the Kaplan–Meier method. Differences were compared with the log-rank test. Cumulative incidence functions for relapse were constructed according to the method reported by Kalbfleisch and Prentice and were compared using Gray's test. The multivariate analysis was conducted using a Cox regression model. Statistical analyses were performed using Graphpad Prism 7, SPSS Statistics 24 and SAS 9.4.

Results

Pediatric B-precursor ALL is associated with late stage differentiation of bone marrow T cells

Relapse rate and bone marrow samples of $n = 100$ pediatric B-precursor ALL patients (pre-B and cALL) were analyzed and compared to bone marrow of healthy donors. All patients received treatment and follow-up in a controlled clinical trial and samples were taken at initial diagnosis prior to ALL treatment. Patient and healthy donor characteristics are summarized in Table 1. Diagnosis of ALL was based on standard criteria including cytomorphology, immune phenotype and percentage of blasts among nucleated cells. Accordingly, T cell analyses and subpopulations are given in frequencies. Differential pre-B-ALL or common-ALL phenotypes had no discernible impact on the interaction with T cells (data not shown) and were therefore summarized as BCP-ALL. Bone marrow of ALL patients was highly infiltrated by $CD10^+/CD19^+$ leukemia blasts (mean 75.1%), with a small population of healthy tissue-resident T cells (bmT) with mean $CD3^+$ of 7.5% vs. 27.1% in healthy individuals (Fig. 1a). Bone marrow T cell subsets showed a significantly reduced percentage of $CD8^+$ T cells in the patients' bone marrow compared to healthy controls' bone marrow T cells (31.6% vs. 48.2%; Fig. 1b). T cell phenotype was markedly affected by leukemic blasts, since the majority of healthy donor bone marrow T cells was assigned to $CD62L^+/CD45RO^-$ T cells like stem cell-like memory (Tscm) or naïve T cell subsets (mean 52.1%), ALL patients' T cells showed a significantly increased percentage of late effector T cell stages (mean 63.8% $CD62L^-/CD45RO^-$ T cells and 25.1% $CD62L^-/CD45RO^+$ effector memory T cells; Fig. 1c). Representative flow cytometry plots illustrating the gating strategy are shown in Supplementary Fig. 1A. Subpopulations characterized by differential expression of CD62L/CD45RO were equally distributed among $CD4^+$ and $CD8^+$ T cells (Supplementary Fig. 1B). Co-stimulatory and co-inhibitory molecules were analyzed by flow cytometry on the surface of $CD10^+/CD19^+$ leukemic blasts and compared to expression levels on $CD10^+/CD19^+$ B-precursor cells in bone marrow of healthy donors. Co-stimulatory surface molecules CD40, CD27, and CD80 as well as co-inhibitory molecules LAG3,



CTLA-4, TIM-3, and PD-L1 were equally expressed in patients and healthy controls (Supplementary Fig. 1C). Exhaustion markers PD-1 and TIM-3 were found to be expressed to the same degree on bmT in healthy individuals and leukemia patients (Fig. 1d). These data indicate that the composition of bmT is associated with late stage

◀ **Fig. 1 Pediatric B-precursor ALL is associated with late stage bone marrow T cell differentiation. a** Frequency of $CD10^+/CD19^+$ B-precursor cells and $CD3^+$ cells in the bone marrow of ALL patients and healthy donors was measured by flow cytometry. **b** B-precursor ALL patients showed a significantly different distribution of $CD4^+$ and $CD8^+$ T cells in the bone marrow. **c** Early and late developmental stages of T cells were analyzed by a simplified two-marker flow cytometry using CD62L and CD45RO, showing significant differences between ALL patients and healthy donors. **d** PD-1 and TIM-3 surface expression on $CD4^+$ and $CD8^+$ bmT was analyzed by flow cytometry. No significant differences between BCP-ALL patients and healthy donors were found. **e** Exemplary flow cytometry plots illustrating the gating strategy for analysis of T cell exhaustion markers. **f** Overall surface expression of PD-1 and TIM-3 was measured by flow cytometry on $CD4^+$ T cells in the bone marrow of ALL patients at initial diagnosis. Patients that will suffer from relapse later in the course of their disease showed a higher overall expression of TIM-3 at time of initial diagnosis of the ALL. Expression of PD-1 showed no significant differences. **g** Representative flow cytometry plot showing TIM-3 expression on $CD4^+$ bmT cells at initial diagnosis of a patient that will have a relapse during follow-up and a patient without relapse during long-term survival. **h** T cell subpopulations among $CD4^+$/TIM-3^+ or $CD4^+$/TIM-3^- bmT cells were analyzed by flow cytometry. Analysis and gating strategy for (**h**) shown in Supplementary Fig. 3; Tn naïve T cells, Tscm stem cell-like memory T cells, Tcm central memory T cells, Tem effector memory T cells, Teff effector T cells, Ttm transitional memory T cells, n.s. not significant, cyPD-L1 cytoplasmic PD-L1. Statistical significance was calculated using a two-tailed *t*-test (CD10, CD19, CD3, CD4, CD8, and T cell phenotypes) or Mann–Whitney test (cyPD-L1); mean with standard deviation (SD) is shown.

differentiation and reduction of early effector stages by the presence of leukemia cells in the bone marrow.

Expression of exhaustion marker TIM-3 on $CD4^+$ bone marrow T cells is a risk factor for relapse of pediatric B-precursor ALL

Diagnostic bone marrow samples of patients with precursor B-ALL were analyzed to identify T cell factors associated with risk of relapse. A follow-up analysis revealed that $n = 86$ of 100 patients survived in complete remission in contrast to $n = 14$ patients who relapsed later in the course of their disease. TIM-3 was significantly overexpressed on $CD4^+$ bone marrow T cells of relapsing patients compared to patients who will survive in complete remission (mean 29.1% vs. 17.9%; Fig. 1e–g). The TIM-3^+CD4^+ T cell population in the bone marrow was further characterized for phenotype of T cell differentiation including CCR7, CD45RO, CD27, CD28, CD127, CD45RA, CD95 CD62L, and CD122 (Supplemental Methods). TIM-3^+CD4^+ T cells showed a shift from naïve T cells to later stages of differentiation. TIM-3^+CD4^+ T cells had significantly less naïve and more transitional memory T cells (Fig. 1h). The majority of $CD4^+$ TIM-3^+ bone marrow T cells was classified as Th1 or Treg cells (Supplementary Figs. 2–4). Although TIM-3 is known to be expressed on Tregs, the frequencies of Tregs did not correlate with relapse of ALL, emphasizing the further analysis of TIM-3 instead of Tregs.

Event-free survival, overall survival of the patient cohort and relapse-free survival (RFS) according to MRD level at end of induction therapy are shown in Fig. 2a–c. The following parameters were analyzed as prognostic factors: T cell differentiation (CD4, CD8, and CD62L/CD45RO proportion), co-stimulation/-inhibition on BCP-ALL (CD27, CD40, CD70, CD80, CD86, CTLA-4, LAG-3, PD-L1, and TIM-3), T cell exhaustion (TIM-3, PD-1, and 2B4). We split the patients in two groups by all possible cutpoints (whole percentages) for each parameter and looked for the parameter/cutpoint with the lowest *p*-value for Gray's test of the cumulative incidence of relapse. These analyses indicated that patients with high TIM-3 expression at timepoint of initial diagnosis (>16% of $CD4^+$ bone marrow T cells) had a significantly higher 6-year cumulative incidence of relapse (pCIR) than patients with low TIM-3 expression (30% SE 8% vs. 5% SE 4%, $p = 0.006\%$; Fig. 2d). The effect is comparable with minimal residual disease at time point 1 (d29) after induction therapy (Fig. 2c). PD-1 expression on $CD4^+$ bone marrow T cells alone did not impact RFS (Fig. 2e), whereas co-expression of PD-1 and TIM-3 (>8.5% of $CD4^+$ bmT cells double positive) was associated with a significantly reduced probability of relapse-free survival (63.1% vs. 89.0%; Fig. 2f). The proportion of double negative (TIM-3^-/PD-1^-) $CD4^+$ T cells in the bone marrow did not influence relapse-free survival (Fig. 2g). To confirm the prognostic relevance of TIM-3 expression on bone marrow T cells, multivariate analysis was performed using COX regression which included the conventional prognostic markers white blood cell count, age, MRD after induction therapy and TIM-3 expression on $CD4^+$ bone marrow T cells as a novel parameter. Multivariate analysis confirmed WBC, age and MRD level as prognostically relevant, although WBC and age did not reach statistical significance in this cohort due to low patient number (Fig. 2h). Multivariate analysis identified TIM-3 expression as statistically significant for relapse risk. The hazard ratio of TIM-3 expression nearly reached the hazard ratio of MRD (7.1 vs. 8.0) indicating that patients with a high TIM-3 expression on $CD4^+$ bone marrow T cells at initial diagnosis have a 7.1-fold increased risk to develop ALL relapse. The distribution of $CD4^+$ and $CD8^+$ T cells as well as the T cell differentiation phenotype (CD62L/CD45RO) showed no significant differences between patients that will remain in complete remission and those relapsing later on. The expression of co-stimulatory and co-inhibitory molecules on $CD8^+$ T cells did not show significant differences in patients that relapsed compared to patients that remained in complete remission. Surface expression of co-stimulation/-inhibition on BCP-ALL (CD27, CD40, CD70, CD80, CD86, CTLA-4, LAG-3,

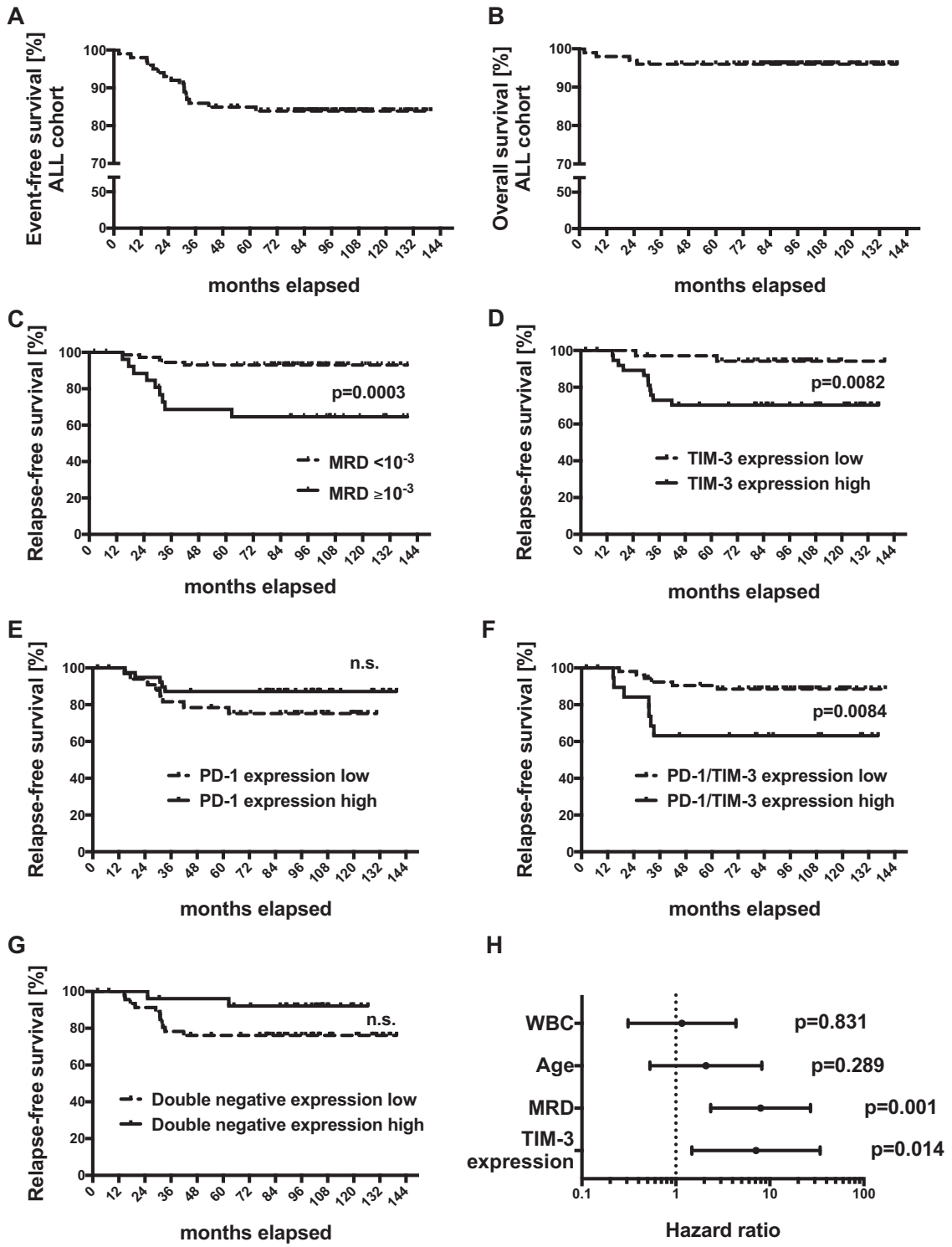


Fig. 2 Expression of exhaustion marker TIM-3 on CD4^{+} bone marrow T cells is a risk factor for relapse of pediatric B-precursor ALL. The ALL cohort showed an **a** event-free and **b** overall survival rate of 84 and 96% respectively. **c** High MRD levels after induction chemotherapy correlates with decreased RFS. Relapse-free survival was additionally evaluated according to **d** the level of overall TIM-3 expression, **e** overall PD-1 expression, **f** PD-1/TIM-3 double expression and **g** in the absence of both markers. Patients with a high TIM-3 overall expression and a high PD-1/TIM-3 double expression showed higher relapse rates compared to patients with low TIM-3 overall or low PD-1/TIM-3 double expression. Statistical significance was calculated using the log-rank (mantel-cox) test. **e** COX regression analysis confirmed an increased relapse hazard ratio (HR) for MRD (HR = 7.98) and TIM-3 expression (HR = 7.12). N.s. not significant, WBC white blood cell count, MRD minimal residual disease.

PD-L1, and TIM-3) did not reach statistical significance between patients that relapsed and patients that remained in complete remission (Supplementary Fig. 1D). Analysis of a validation cohort ($n = 40$; CI at 6 years follow-up) confirmed the significant increase ($p = 0.03$) of the cumulative risk of relapse in children with a high frequency of TIM-3$^+$CD4$^+$ bone marrow T cells (Supplementary Fig. 5). The overall numbers of TIM-3$^+$ cells were slightly different in the initial cohort compared to the validation cohort. This led to a different threshold for high TIM-3$^+$CD4$^+$ bone marrow T cells (16% vs. 10%).

Leukemia associated upregulation of TIM-3 on T cells leading to reduced activation and proliferation potential of T cells

To investigate the functional role of TIM-3 in anti-leukemic T cell responses, retroviral TIM-3 overexpression and CRISPR/Cas9-mediated TIM-3 knockout (KO) were performed by ribonucleoprotein electroporation into primary T cells. Mean TIM-3 transduction rates of 40.2% were achieved (Supplementary Fig. 6A). CRISPR/Cas9-mediated TIM-3 KO rates were analyzed by flow cytometry and DNA sequencing and showed mean KO rates of 82.7% (protein level) and 58.6% (genomic DNA level) (Supplementary Fig. 6B). TIM-3 wild type and TIM-3 KO T cells were analyzed with cells from different individuals in multiple independent experiments and are summarized in Fig. 3. Analysis included activation marker, proliferation and TIM-3 surface expression after co-culture with CD19$^+$ leukemic cell lines with or without the bi-specific T cell engager (BiTE) blinatumomab to induce direct cell-to-cell contact and a CD3/CD19-mediated T cell response against leukemic cells. Wild type T cells showed a significant upregulation of TIM-3 upon induction of an anti-leukemic T cell response (Fig. 3a). To analyze the functional relevance of TIM-3 in this model, TIM-3 KO or TIM-3 overexpressing and wild type T cells were co-cultured with staphylococcal enterotoxin B (SEB) or with CD19$^+$ leukemic cells in the presence of blinatumomab. Levels of T cell activation markers CD69 and 4-1BB were measured by flow cytometry. Exemplary flow cytometry plots of 4-1BB levels after stimulation of TIM-3 KO and overexpressing cells vs controls are shown in Fig. 3b. Surface expression of CD69 and 4-1BB after a 24-h co-culture was compared to background expression of T cells and normalized to control T cells. TIM-3 KO cells showed a significant increase in expression of activation markers compared to wild type T cells (Fig. 3c, d), whereas TIM-3 overexpressing cells showed a decrease, although not statistically significant (Supplementary Fig. 6C). Cell activation induced by co-culture with SEB showed similar effects as a positive control (Fig. 3c, d and Supplementary Fig. 6C). These data confirm that TIM-3 expression impairs leukemia-induced T cell activation, whereas basic T cell function such as cytotoxicity after 24 h is preserved (Supplementary Fig. 6D & E). To investigate TIM-3 effects on T cell proliferation, TIM-3 KO and wild type T cells were co-cultured with CD19$^+$ target cells and blinatumomab (Fig. 3e, left panel) or stimulated with SEB (Fig. 3e, right panel). The proportion of proliferating T cells after 96 h was analyzed by flow cytometry, compared to percentage of background proliferating T cells and normalized to wild type control T cells. TIM-3 KO cells showed an increase of proliferating T cells compared to wild type T cells. When TIM-3 overexpressing T cells were co-cultured with CD19$^+$ target cells (Fig. 3f, left panel) or stimulated with SEB (Fig. 3f, right panel), they showed a significantly decreased proliferation compared to wild type T cells. These findings confirm that TIM-3 expression impairs T cell activation and proliferation of leukemia-mediated T cell responses.

ALL-induced TIM-3 expression on CD4$^+$ T cells is mediated by CD200

RNA next generation sequencing was performed to analyze differences between the groups of TIM-3 high expressing ($n = 12$) and TIM-3 low expressing ALL samples ($n = 15$). All diagnostic bone marrow samples consisted of >70% leukemic blasts. RNA sequencing revealed a low number of differentially expressed genes between the two groups (Supplementary Fig. 7A). Expression of known TIM-3 ligands was analyzed to examine possibility of downstream activity. *HMGB1*, galectin-9 (gene name: *LGALS9*) and *CEACAM1* were identified by RNA sequencing (Fig. 4a). Known TIM-3 inducers such as IL-2 (gene name*:* *IL2*), IL-12 (*IL12A/B*), IL-7 (*IL7*) and IL-15 (*IL15*), IL-21 (*IL21*), IL-27 (*IL27*), and TGF-β (*TGFB1*) were either not expressed on RNA level or they were not differentially expressed between the two groups (Fig. 4b). These observations suggest that TIM-3 expression on CD4$^+$ bmT cells was not caused by proinflammatory signals that are currently known to induce TIM-3, but might be mediated by a different mechanism of interaction with ALL blasts.

To evaluate whether known co-stimulatory/-inhibitory molecules could be involved in TIM-3 upregulation, statistical analysis was confined to 21 pre-defined known immune-modulatory molecules (Supplementary Table 2). In this analysis, the co-inhibitory immune checkpoint *CD200* was found to be significantly overexpressed ($p = 0.0005$, adjusted $p = 0.0096$) in patient samples with high percentage of TIM-3$^+$ CD4$^+$ bone marrow T cells (Fig. 4c). None of the remaining co-stimulatory (Supplementary Fig. 7B) or co-inhibitory molecules (Supplementary Fig. 7C) was differentially expressed, hence CD200 was further analyzed. In order to dissect the effect of TIM-3 and PD-1 we analyzed

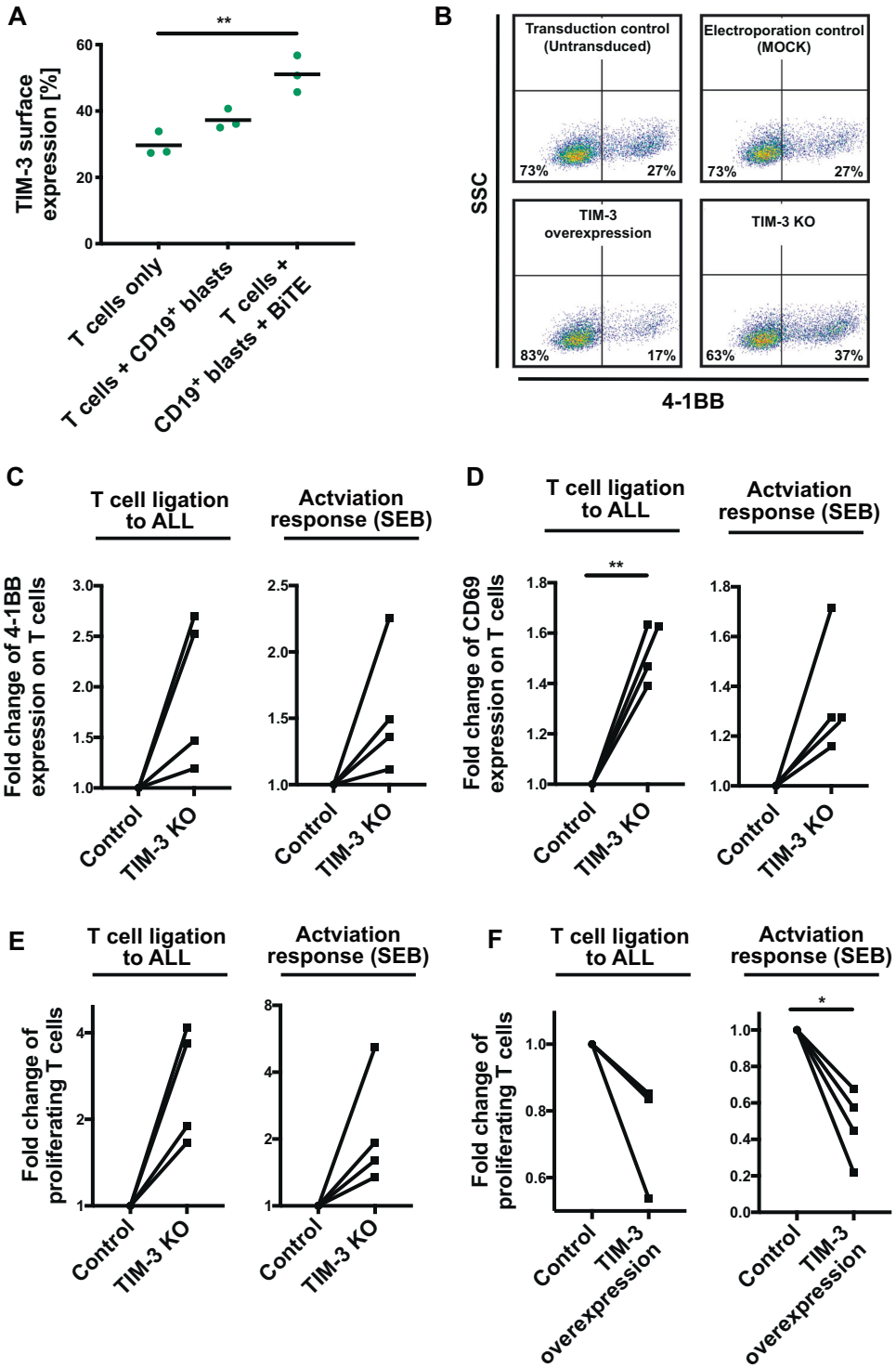
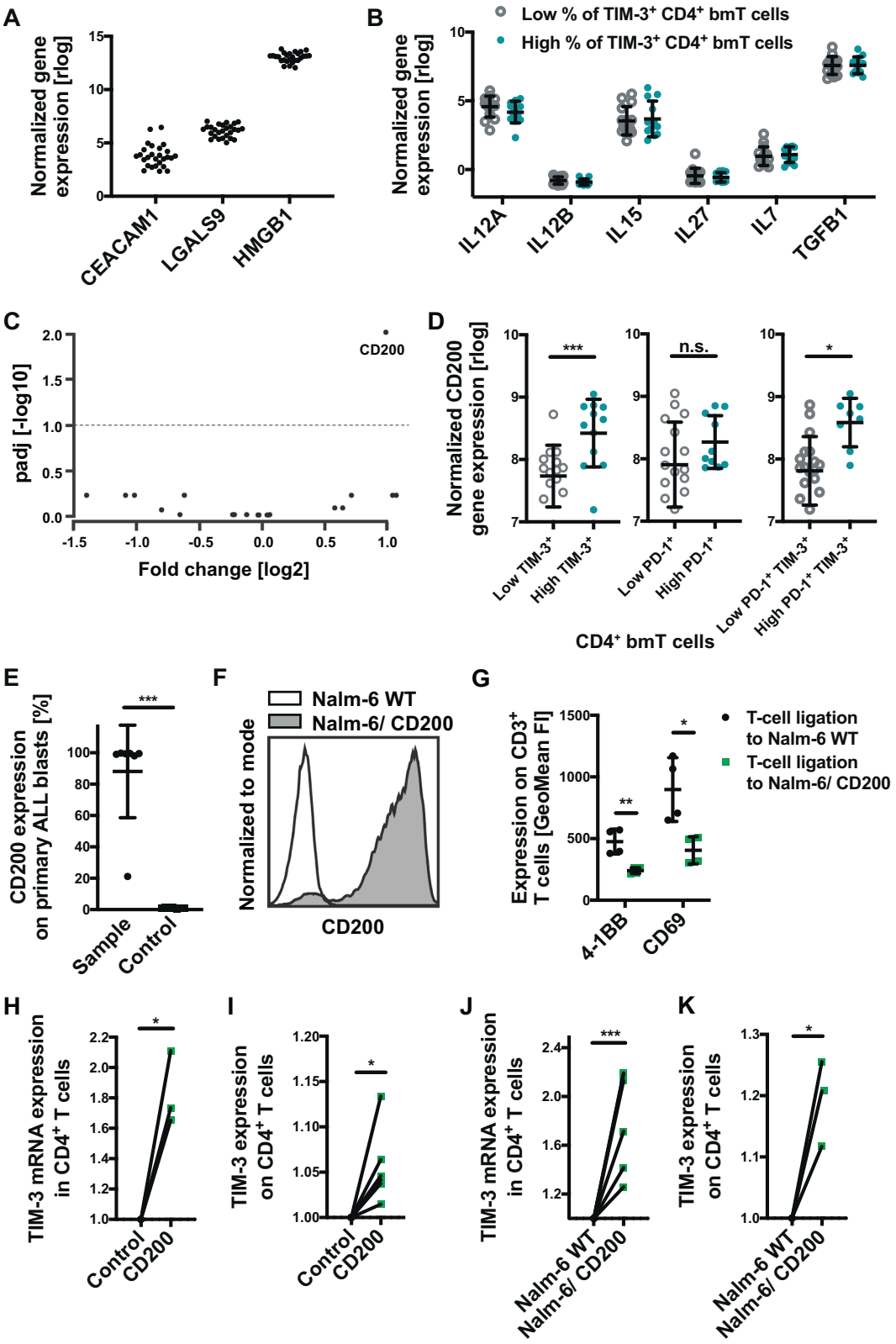


Fig. 3 Leukemia is associated with upregulation of TIM-3 on T cells leading to reduced activation and proliferation potential of T cells. a T cells were left untreated or co-cultured with CD19^{+} target cells with/without blinatumomab that causes a direct T cell to blast contact. TIM-3 expression is upregulated in response to contact to ALL blasts after 24 h ($n = 3$; bar represents mean). **b** CRISPR/Cas9-mediated TIM-3 knockout and retroviral overexpression of TIM-3 in primary T cells were performed. Expression of the activation marker 4-1BB was measured in TIM-3 KO and overexpressing samples upon stimulation with SEB. **c**, **d** Control T cells and T cells with TIM-3 KO were co-cultured with CD19^{+} targets and blinatumomab (left panel) or activated with SEB (right panel). CD69 and 4-1BB surface expression was analyzed after 24 h. Fold change of surface expression is shown and compared to control T cells. **e** Control and TIM-3 KO T cells were co-cultured with CD19^{+} targets and blinatumomab (left panel) or activated with SEB (right panel). T cell proliferation after 72 h was analyzed by flow cytometry. **f** Control and TIM-3 overexpressing T cells were co-cultured with CD19^{+} targets and blinatumomab (left panel) or activated with SEB (right panel). T cell proliferation after 72 h was analyzed by flow cytometry. Proliferation fold change compared to control T cells is shown. Significance was calculated using paired *t*-test. BiTE bispecific T cell engager blinatumomab, SEB staphylococcal enterotoxin B.

differential CD200 expression in ALLs in the subgroups with high or low percentage of TIM-3^{+} and/or PD-1^{+} bmT (Fig. 4d). We confirmed the correlation of CD200 in ALL blasts and TIM-3 on bmT cells, since only patients with high percentage of TIM-3^{+} and TIM-3^{+}/PD-1^{+} bmT cells had a significantly increased *CD200* RNA level in their ALL blasts. Contrarily, bone marrow samples with PD-1^{+} bmT only, did not show increased CD200 RNA levels (Fig. 4d). High CD200 surface expression was confirmed by flow cytometry on primary BCP-ALL blasts compared to healthy bone marrow BCP cells as controls (Fig. 4e). To investigate the functional impact of CD200 on T cell activation, BCP-ALL cells (Nalm-6; wild type has no CD200 expression) were retrovirally transduced with human



CD200 (Fig. 4f). T cell response was induced against either Nalm-6 WT or Nalm-6/CD200$^+$ using blinatumomab. Nalm-6/CD200$^+$ significantly decreased T cell activation compared to wild type ALL cells (Fig. 4g). To identify the impact of CD200 on TIM-3 expression levels, primary T cells were cultured on CD200 Fc chimera-coated plates.

◀ **Fig. 4 ALL-induced upregulation of TIM-3 on CD4$^+$ T cells is mediated by CD200. a** RNA sequencing of 27 ALL samples was performed. Normalized counts (log2) of TIM-3 ligands *CEACAM1*, galectin-9 (*LGALS9*) and *HMGB1* are shown. **b** Expression of TIM-3 inducers IL-12 (*IL12A/B*), IL-15 (*IL15*), IL-27 (*IL27*), IL-7 (*IL7*), and TGF-β (*TGFB1*) was compared between patients with high ($n = 12$)/ low ($n = 15$) TIM-3 expression. **c** Volcano plot of 21 immune-modulatory genes investigated on TIM-3 high/low expressing samples. Adjusted *p* value and expression fold change (TIM-3 high/low expressing samples) were calculated. **d** CD200 gene expression in samples with high/low frequency of TIM-3$^+$ CD4$^+$ bmT cells, high/ low PD-1$^+$CD4$^+$ bmT cells and high/low double positive CD4$^+$ bmT cells. **e** Surface expression of CD200 was analyzed on primary ALL blasts ($n = 7$) compared to healthy bone marrow BCP cells. **f** Nalm-6 cells were transduced with CD200. **g** T cells were co-cultured with blinatumomab and Nalm-6 WT or Nalm-6/CD200 cells. Co-culture with Nalm-6/CD200 led to decreased 4-1BB and CD69 expression analyzed by flow cytometry. **h** T cells were cultured on CD200-coated plates. TIM-3 expression on CD4$^+$ T cells was analyzed 24 h later by qPCR and **i** flow cytometry (geometric mean fluorescence intensity), $n \geq 3$ individual donors. **j** T cells were co-cultured with Nalm-6/ CD200. TIM-3 expression on CD4$^+$ T cells was analyzed 24–72 h later by qPCR and **k** flow cytometry, $n \geq 3$ individual donors. Paired *t*-test was performed in **g**–**k**, DESeq2 normalization is shown for **a**, **b**, and **d**. Padj = adjusted *p* value; n.s. not significant, WT wild type. Mean with SD is shown.

TIM-3 expression was measured after 24 h and compared to T cells cultured on isotype control-coated plates. Coating with CD200 induced significant upregulation of TIM-3 on CD4$^+$ T cells measured by quantitative PCR (qPCR) (Fig. 4h) and flow cytometry (Fig. 4i). To prove this effect on leukemic cell lines, primary T cells were co-cultured with CD200-overexpressing ALL cells. TIM-3 expression on CD4$^+$ T cells was analyzed after 24–72 h of co-culture. The CD200-transduced leukemic cells significantly induced TIM-3 expression on CD4$^+$ T cells measured by qPCR and flow cytometry (Fig. 4j, k) confirming TIM-3 induction on CD4$^+$ T cells by CD200 expression on leukemic cells in three independent experiments from at least three individuals.

Discussion

BCP-ALL of childhood has been the most important target malignancy for emerging immunotherapies like bispecific antibodies or chimeric antigen receptor T cells. However, insufficient activation, in vivo expansion and persistence of T cells has limited success of T cell immunotherapy. Interaction of BCP-ALL cells with bmT cells is rather likely, since these malignant cells derive from antigen-presenting cells and have numerous cellular interactions with T cells. Therefore, markers of T cell dysfunction in the bone marrow may broaden the understanding of the disease and open future options of immunotherapy. Based on previous findings that leukemia cells are able to upregulate co-inhibitory molecules such as PD-L1 after exposure to Th1 responses [27], we analyzed expression profiles of known co-inhibitory and co-stimulatory molecules on a large cohort of BCP-ALL. In a first step, molecules involved in co-inhibition and co-stimulation were compared to healthy controls and in a second step, we investigated the prognostic relevance of immunological markers. All patients were treated in a prospective clinical study and underwent systematic follow-up with a median follow-up time of 7.8 years.

The comparison between bmT cells in healthy individuals and pediatric BCP-ALL showed that pediatric B-precursor ALL is associated with a terminally differentiated T cell phenotype and significant reduction of CD8$^+$ cells. Based on these findings, we defined bmT cells of ALL patients as tumor-infiltrating lymphocytes. The analysis of bmT describes the situation at the time of diagnosis, but does not prove the underlying pathogenesis of these changes. The described bmT could be either resident cells driven into late stage differentiation by the ALL blasts or otherwise, the bmT could be a different population of cells recruited in response to the malignancy. However, the concept of immunosurveillance and tumor immune escape includes exhaustion of T cells. In murine models [28] and after allogeneic stem cell transplantation [29] ALL has been associated with T cells expressing PD-1 and TIM-3. The negative regulation of T cell activity by TIM-3 is well known in health and solitary diseases [30]. Therefore, we analyzed expression levels of inhibitory exhaustion markers PD-1 and TIM-3 on bmT cells. TIM-3 expression and combined TIM-3/PD-1 expression on CD4$^+$ bmT cells were identified as strong prognostic factors for relapse-free survival within the BCP-ALL patient cohort. This finding was confirmed in a validation cohort. PD-1 expression on T cells alone and expression levels on CD8$^+$ T cells had no impact on relapse-free survival rates. Multivariate COX regression analysis including conventional prognostic markers like MRD confirmed strong prognostic relevance for increase of TIM-3$^+$CD4$^+$ bmT cells. Patients with increase of TIM-3$^+$CD4$^+$ bmT cells had a 7.1-fold higher relapse risk (hazard ratio) compared to patients with low numbers of TIM-3$^+$CD4$^+$ bmT cells. Notably, hazard ratios for increase of TIM-3$^+$CD4$^+$ bmT cells nearly reached those of conventional marker MRD after induction therapy, which is currently the strongest marker of relapse risk in ALL treatment protocols [31]. This finding is in accordance with recently published data in adult patients suffering from ALL, showing that the proportion of PD1$^+$TIM-3$^+$ double-positive CD4$^+$ T cells differentiated a poor survival group [25]. The TIM-3$^+$CD4$^+$ T cell population in the bone marrow was further characterized to exclude the error that TIM-3 is an epiphenomena of an underlying T cell subpopulation. Naïve T cells were significantly decreased in TIM-3$^+$CD4$^+$ bmT cells compared to TIM-3$^-$ T cells.

Transitional memory T cells were significantly increased in TIM-3$^+$CD4$^+$ bmT cells compared to TIM-3$^-$ T cells. This is in accordance with the described finding that the presence of ALL blasts shifts the composition of subpopulations from naïve stages to later stages of differentiation. Both findings are in line with the assumption that ALL induces changes in bone marrow T cells. In addition, we analyzed Tregs and Th1, Th2 cell subpopulations. There was no single subpopulation that outcompeted TIM-3 as a marker of relapse risk. This fact underlines the relevance of TIM-3 as a new marker.

In vitro experiments confirmed that TIM-3 is upregulated on primary T cells after contact with leukemic blasts and during leukemia-specific T cell attack. To study the effect of TIM-3 upregulation and downregulation in this leukemia model, retroviral overexpression and CRISPR/Cas9-based TIM-3 KO in primary T cells were performed. Co-culture experiments confirmed that TIM-3 expression decreases activation and proliferation potential of anti-leukemic T cell responses. Based on these findings, we conclude that the non-specific effect of TIM-3 involving activation and proliferation processes is relevant for the interaction between T cells and ALL blasts. TIM-3 is known as an unspecific marker associated with decrease of T cell function and is not restricted to the specificity of the T cell receptor (TCR). In our study we did not investigate the variable regions of the TCR, since an effect of the ALL blasts on the surrounding microenvironment is supposed to affect T cells with different TCRs.

Next, we aimed to identify mechanisms in BCP-ALL that mediate or induce TIM-3 on bmT cells. Known binding partners of TIM-3 are HMGB1, galectin-9, and CEACAM1 [13–15, 22]. Presence of all three ligands confirmed that TIM-3 can exert its activity and has receptors on ALL blasts. RNA-seq data of the primary ALL blasts revealed that presence or absence of ligands does not account for the prognostic difference inferred by TIM-3 expression on CD4$^+$ bmT cells. In a healthy immune system, TIM-3 is upregulated strongly on differentiated Th1 cells and induced by inflammatory cytokines like IL-7, IL-12, IL-15 as well as by immunosuppressive cytokines like IL-27 [30]. Known inflammatory TIM-3 inducers were analyzed by RNA expression levels [9–12]. Known TIM-3 inducers were not associated with increase of TIM-3$^+$CD4$^+$ bmT cells. Hence, we searched for potentially novel TIM-3 inducers and a mechanism of TIM-3 induction. Multiple testing analysis was confined to selected immune-modulatory molecules ($n = 21$). Among these CD200 was identified in the primary bone marrow samples to be significantly upregulated in samples with increase of TIM-3$^+$CD4$^+$ bmT cells. None of the other tested immune checkpoint molecules was differentially expressed. CD200 is a type Ia transmembrane protein expressed on lymphoid and neuronal tissue [32]. Overexpression of CD200 was previously observed in a variety of cancer entities including melanoma, squamous cell carcinoma, chronic lymphocytic leukemia (CLL) and acute myeloid leukemia (AML) [33–39] and especially pediatric ALL cells express high levels of CD200 [27]. Its receptor, CD200R, is expressed on APCs and T cells [33]. CD200/CD200R interaction inhibits IL-2 and IFN-γ production by monocytes and macrophages and decreases T cell mediated responses by induction of regulatory T cells (Tregs) [40, 41]. An interaction between CD200 and TIM-3 has not been previously described. We therefore analyzed how CD200 on ALL cells changes the activation of T cells and confirmed significant impairment of T cell activation mediated by CD200. In addition, TIM-3 expression on CD4$^+$ T cells could be induced by CD200 in vitro. However, our in vitro data do not include the specific microenvironment of the bone marrow niche and therefore the effect of bystander cells remains to be investigated.

In conclusion, our data show that ALL is associated with a composition of bone marrow T cells shifted to late effector differentiation stages and reduced frequencies of cytotoxic T cells. In those patients with overexpression of an exhausted TIM-3$^+$ CD4$^+$ bmT cell-phenotype, the risk of relapse is significantly increased. Here we identify CD200 as a co-factor for TIM-3-mediated suppression of T cell function. The TIM-3/CD200 axis constitutes a mechanism of immune dysregulation in BCP-ALL. Future studies will evaluate whether TIM-3 expression also influences outcome of immunotherapeutic approaches such as blinatumomab or CAR T cell treatment. Targeting the TIM-3/CD200 axis might be a useful approach to improve current treatment strategies.

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Author contributions The concept was set up by T.F. Design and approach of experiments was done by F.B. and T.F. Diagnostic characterization and follow-up analyses were performed by M.H. and G.E. Statistics were done by M.Z. RNA sequencing was done by M.R., S.W., and C.K. V.B. provided patient and healthy donor samples. TIM-3 and CD200 experiments were done by F.B., M.L., D.S., S. W., and T.K. Bioinformatics were done by F.R.R. and S.C. Data analysis and manuscript preparation was done by F.B. and T.F. The manuscript was reviewed by all authors.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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