From the: Dr. von Haunersches Kinderspital,

Ludwig-Maximilians-Universität zu München

Dissertation

zum Erwerb des Doctor of Philosophy (Ph.D.) an der

Medizinischen Fakultät der

Ludwig-Maximilians-Universität zu München

Immunotherapy of pediatric acute lymphoblastic B-cell leukemia using gene-modified T cells

vorgelegt von:

Dr. med. Franziska Blaeschke

aus:

Darmstadt, Deutschland

Jahr:

2021

Mit Genehmigung der Medizinischen Fakultät der

Ludwig-Maximilians-Universität zu München

First supervisor: *Prof. Dr. med. Tobias Feuchtinger*

Second supervisor*:* *Prof. Dr. med. Sebastian Kobold*

Third supervisor: *Prof. Dr. med. Dirk H. Busch*

Dean: **Prof. Dr. med. dent. Reinhard Hickel**

Datum der Verteidigung:

13.09.2021

Affidavit

LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

Dean's Office
Medical Faculty

MMRS

Affidavit

Blaeschke, Franziska
Surname, first name

Dr. von Haunersches Kinderspital, Lindwurmstrasse 4
Street

80337 Muenchen
Zip code, town

Germany
Country

I hereby declare, that the submitted thesis entitled

Immunotherapy of pediatric acute lymphoblastic B-cell leukemia using gene-modified T cells

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

San Francisco, September 26, 2021
Place, date

Franziska Blaeschke
Signature doctoral candidate

Confirmation of congruency

LMU LUDWIG-MAXIMILIANS-UNIVERSITÄT MÜNCHEN

Dean's Office
Medical Faculty

MMRS

Confirmation of congruency between printed and electronic version of the doctoral thesis

Blaeschke, Franziska
Surname, first name

Dr. von Haunersches Kinderspital, Lindwurmstrasse 4
Street

80337 Muenchen
Zip code, town

Germany
Country

I hereby declare that the electronic version of the submitted thesis, entitled
Immunotherapy of pediatric acute lymphoblastic B-cell leukemia using gene-modified T cells

is congruent with the printed version both in content and format.

San Francisco, September 26, 2021
Place, date

Franziska Blaeschke
Signature doctoral candidate

Table of content

List of abbreviations

AML	Acute myeloid leukemia
APC	Antigen-presenting cell
BCP-ALL	B-cell precursor acute lymphoblastic leukemia
CAR	Chimeric antigen receptor
Cas9	CRISPR-associated protein 9
CD	Cluster of differentiation
CD200R	CD200 receptor
CEACAM-1	Carcinoembryonic antigen-related cell adhesion molecule 1
CRISPR	Clusters of regularly interspaced short palindromic repeats
CRS	Cytokine release syndrome
EMA	European Medicines Agency
FDA	U.S. Food and Drug Administration
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GMP	Good Manufacturing Practice
gRNA	Guide RNA
HMGB1	High mobility group box 1
IFN-γ	Interferon gamma
IL	Interleukin
KO	Knockout
MRD	Minimal residual disease
PD-1	Programmed cell death protein 1
PtdSer	Phosphatidylserine
scFv	Single-chain variable fragment
TCR	T-cell receptor
TGF-β	Transforming Growth Factor beta
Th	T helper
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TNF-α	Tumor Necrosis Factor alpha

List of publications

1. Willier S, Rothamel P, Hastreiter M, Wilhelm J, Stenger D, **Blaeschke F**, Rohlfs M, Kaeuferle T, Schmid I, Albert MH, Binder V, Subklewe M, Klein C, Feuchtinger T. 2021. CLEC12A and CD33 co-expression as preferential target on pediatric AML for combinatorial immunotherapy. Blood.

2. Rutishauser RL, Deguit CDT, Hiatt J, **Blaeschke F**, Roth TL, Wang L, Raymond KA, Starke CE, Mudd JC, Chen W, Smullin CP, Matus-Nicodemos R, Hoh R, Krone MR, Hecht FM, Pilcher CD, Martin JN, Koup RA, Douek DC, Brenchley JM, Sekaly RP, Pillai SK, Marson A, Deeks SG, McCune JM, Hunt PW. 2021. TCF-1 regulates HIV-specific CD8+ T cell expansion capacity. JCI Insight.

3. Stenger D, Stief TA, Kaeuferle T, Willier S, Rataj F, Schober K, Vick B, Lotfi R, Wagner B, Grunewald TGP, Kobold S, Busch DH, Jeremias I, **Blaeschke F***, Feuchtinger T*. 2020. Endogenous TCR promotes in vivo persistence of CD19-CAR-T cells compared to a CRISPR/Cas9-mediated TCR knockout CAR. Blood;136(12):1407-18. (*These authors contributed equally.)

4. Willier S, Raedler J, **Blaeschke F**, Stenger D, Pazos Escudero M, Jurgeleit F, Grunewald TGP, Binder V, Schmid I, Albert MH, Wolf A, Feuchtinger T. 2020. Leukemia escape in immune desert: intraocular relapse of pediatric pro-B-ALL during systemic control by CD19-CAR T cells. J Immunother Cancer;8(2).

5. Roth TL, Li PJ*, **Blaeschke F***, Nies JF*, Apathy R*, Mowery C*, Yu R, Nguyen MLT, Lee Y, Truong A, Hiatt J, Wu D, Nguyen DN, Goodman D, Bluestone JA, Ye CJ, Roybal K, Shifrut E, Marson A. 2020. Pooled Knockin Targeting for Genome Engineering of Cellular Immunotherapies. Cell;181(3):728-44 e21. (*These authors contributed equally.)

6. Kaeuferle T, Deisenberger L, Jablonowski L, Stief TA, **Blaeschke F**, Willier S, Feuchtinger T. 2020. CRISPR-Cas9-Mediated Glucocorticoid Resistance in Virus-Specific T Cells for Adoptive T Cell Therapy Posttransplantation. Mol Ther;28(9):1965-73.

7. Dong R, Libby KA, **Blaeschke F**, Fuchs W, Marson A, Vale RD, Su X. 2020. Rewired signaling network in T cells expressing the chimeric antigen receptor (CAR). EMBO J;39(16):e104730.

8. **Blaeschke F**, Willier S, Stenger D, Lepenies M, Horstmann MA, Escherich G, Zimmermann M, Rojas Ringeling F, Canzar S, Kaeuferle T, Rohlfs M, Binder V, Klein C, Feuchtinger T. 2020. Leukemia-induced dysfunctional TIM-3(+)CD4(+) bone marrow T cells increase risk of relapse in pediatric B-precursor ALL patients. Leukemia;34(10):2607-20.

9. Willier S, Cabanillas Stanchi KM, von Have M, Binder V, **Blaeschke F**, Feucht J, Feuchtinger T, Doring M. 2019. Efficacy, safety and feasibility of fosaprepitant for the prevention of chemotherapy-induced nausea and vomiting in pediatric patients receiving moderately and highly emetogenic chemotherapy - results of a non-interventional observation study. BMC Cancer;19(1):1118.

10. Kaeuferle T, Krauss R, **Blaeschke F**, Willier S, Feuchtinger T. 2019. Strategies of adoptive T -cell transfer to treat refractory viral infections post allogeneic stem cell transplantation. J Hematol Oncol;12(1):13.

11. Calderon D, Nguyen MLT, Mezger A, Kathiria A, Muller F, Nguyen V, Lescano N, Wu B, Trombetta J, Ribado JV, Knowles DA, Gao Z, **Blaeschke F**, Parent AV, Burt TD, Anderson MS, Criswell LA, Greenleaf WJ, Marson A, Pritchard JK. 2019. Landscape of stimulation-responsive chromatin across diverse human immune cells. Nat Genet;51(10):1494-505.

12. **Blaeschke F**, Paul MC, Schuhmann MU, Rabsteyn A, Schroeder C, Casadei N, Matthes J, Mohr C, Lotfi R, Wagner B, Kaeuferle T, Feucht J, Willier S, Handgretinger R, Stevanovi CS, Lang P, Feuchtinger T. 2019. Low mutational load in pediatric medulloblastoma still translates into neoantigens as targets for specific T-cell immunotherapy. Cytotherapy;21(9):973-86.

13. **Blaeschke F**, Stenger D, Kaeuferle T, Willier S, Lotfi R, Kaiser AD, Assenmacher M, Doring M, Feucht J, Feuchtinger T. 2018. Induction of a central memory and stem cell memory phenotype in functionally active CD4(+) and CD8(+) CAR T cells produced in an automated good manufacturing practice system for the treatment of CD19(+) acute lymphoblastic leukemia. Cancer Immunol Immunother;67(7):1053-66.

14. Baldauf MC, Gerke JS, Kirschner A, **Blaeschke F**, Effenberger M, Schober K, Rubio RA, Kanaseki T, Kiran MM, Dallmayer M, Musa J, Akpolat N, Akatli AN, Rosman FC, Ozen O, Sugita S, Hasegawa T, Sugimura H, Baumhoer D, Knott MML, Sannino G, Marchetto A, Li J, Busch DH, Feuchtinger T, Ohmura S, Orth MF, Thiel U, Kirchner T, Grunewald TGP. 2018. Systematic identification of cancer-specific MHC-binding peptides with RAVEN. Oncoimmunology;7(9):e1481558.

15. Thiel U, Schober SJ, Einspieler I, Kirschner A, Thiede M, Schirmer D, Gall K, **Blaeschke F**, Schmidt O, Jabar S, Ranft A, Alba Rubio R, Dirksen U, Grunewald TGP, Sorensen PH, Richter GHS, von Luttichau IT, Busch DH, Burdach SEG. 2017. Ewing sarcoma partial regression without GvHD by chondromodulin-I/HLA-A*02:01-specific allorestricted T cell receptor transgenic T cells. Oncoimmunology;6(5):e1312239.

16. Doring M, Cabanillas Stanchi KM, Queudeville M, Feucht J, **Blaeschke F**, Schlegel P, Feuchtinger T, Lang P, Muller I, Handgretinger R, Heinz WJ. 2017. Efficacy, safety and feasibility of antifungal prophylaxis with posaconazole tablet in paediatric patients after haematopoietic stem cell transplantation. J Cancer Res Clin Oncol;143(7):1281-92.

17. Doring M, Cabanillas Stanchi KM, Klinker H, Eikemeier M, Feucht J, **Blaeschke F**, Schwarze CP, Ebinger M, Feuchtinger T, Handgretinger R, Heinz WJ. 2017. Posaconazole plasma concentrations in pediatric patients receiving antifungal prophylaxis during neutropenia. Med Mycol;55(4):375-84.

18. Boekstegers AM, **Blaeschke F**, Schmid I, Wiebking V, Immler S, Hoffmann F, Bochmann K, Muller S, Grunewald TGP, Feucht J, Feuchtinger T. 2017. MRD response in a refractory paediatric T-ALL patient through anti-programmed cell death 1 (PD-1) Ab treatment associated with induction of fatal GvHD. Bone Marrow Transplant;52(8):1221-4.

19. Thiel U, Wawer A, von Luettichau I, Bender HU, **Blaeschke F**, Grunewald TG, Steinborn M, Roper B, Bonig H, Klingebiel T, Bader P, Koscielniak E, Paulussen M, Dirksen U, Juergens H, Kolb HJ, Burdach SE. 2016. Bone marrow involvement identifies a subgroup of advanced Ewing sarcoma patients with fatal outcome irrespective of therapy in contrast to curable patients with multiple bone metastases but unaffected marrow. Oncotarget;7(43):70959-68.

20. Kirschner A, Thiede M, **Blaeschke F**, Richter GH, Gerke JS, Baldauf MC, Grunewald TG, Busch DH, Burdach S, Thiel U. 2016. Lysosome-associated membrane glycoprotein 1 predicts

fratricide amongst T cell receptor transgenic CD8+ T cells directed against tumor-associated antigens. Oncotarget;7(35):56584-97.

21. Feucht J, Kayser S, Gorodezki D, Hamieh M, Doring M, **Blaeschke F**, Schlegel P, Bosmuller H, Quintanilla-Fend L, Ebinger M, Lang P, Handgretinger R, Feuchtinger T. 2016. T-cell responses against CD19+ pediatric acute lymphoblastic leukemia mediated by bispecific T-cell engager (BiTE) are regulated contrarily by PD-L1 and CD80/CD86 on leukemic blasts. Oncotarget;7(47):76902-19.

22. **Blaeschke F***, Thiel U*, Kirschner A, Thiede M, Rubio RA, Schirmer D, Kirchner T, Richter GHS, Mall S, Klar R, Riddell S, Busch DH, Krackhardt A, Grunewald TG, Burdach S. 2016. Human HLA-A*02:01/CHM1+ allo-restricted T cell receptor transgenic CD8+ T cells specifically inhibit Ewing sarcoma growth in vitro and in vivo. Oncotarget;7(28):43267-80. (*These authors contributed equally.)

23. Thiel U, Koscielniak E, **Blaeschke F**, Grunewald TG, Badoglio M, Diaz MA, Paillard C, Prete A, Ussowicz M, Lang P, Fagioli F, Lutz P, Ehninger G, Schneider P, Santucci A, Bader P, Gruhn B, Faraci M, Antunovic P, Styczynski J, Kruger WH, Castagna L, Rohrlich P, Ouachee-Chardin M, Salmon A, Peters C, Bregni M, Burdach S, Solid Tumour Working P, the Paediatric Disease Working Party of the European Group for B, Marrow T. 2013. Allogeneic stem cell transplantation for patients with advanced rhabdomyosarcoma: a retrospective assessment. Br J Cancer;109(10):2523-32.

24. Thiel U, Wolf P, Wawer A, **Blaeschke F**, Grunewald TG, von Luttichau IT, Klingebiel T, Bader P, Borkhardt A, Laws HJ, Handgretinger R, Lang P, Schlegel PG, Eyrich M, Gruhn B, Ehninger G, Koscielniak E, Klein C, Sykora KW, Holler E, Mauz-Korholz C, Woessmann W, Richter GH, Schmidt AH, Peters C, Dirksen U, Jurgens H, Bregni M, Burdach S. 2012. Human leukocyte antigen distribution in German Caucasians with advanced Ewing's sarcoma. Klin Padiatr;224(6):353-8.

Contribution to the publications

1.1 Contribution to paper I

Cancer Immunology, Immunotherapy (2018) 67:1053–1066
https://doi.org/10.1007/s00262-018-2155-7

ORIGINAL ARTICLE

Induction of a central memory and stem cell memory phenotype in functionally active CD4$^+$ and CD8$^+$ CAR T cells produced in an automated good manufacturing practice system for the treatment of CD19$^+$ acute lymphoblastic leukemia

Franziska Blaeschke[1] · Dana Stenger[1] · Theresa Kaeuferle[1] · Semjon Willier[1] · Ramin Lotfi[2,3] · Andrew Didier Kaiser[4] · Mario Assenmacher[4] · Michaela Döring[1,5] · Judith Feucht[5,6] · Tobias Feuchtinger[1]

I hereby confirm that I

- Drafted the proposal for the institutional ethical review board and all patient consent agreements.
- Wrote funding grant (Bettina Braeu Stiftung).
- Consented patients for the study.
- Drafted the scientific part of the research agreement between Miltenyi Biotec and the Dr. von Hauner Children's Hospital.
- Designed the approach of the study together with Tobias Feuchtinger.
- Received training on the CliniMACS Prodigy at Miltenyi Biotec.
- Designed the experiments together with Tobias Feuchtinger and Andrew Didier Kaiser.
- Performed CliniMACS Prodigy runs CAR001 and CAR002 at Miltenyi Biotec (supervised by their team) including lentivirus production and data analysis.
- Transferred the process from Miltenyi Biotec to the Dr. von Hauner Children's Hospital and trained staff from our lab.
- Performed CliniMACS Prodigy runs CAR003 and CAR004 together with Dana Stenger (supported by technicians from our lab).
- Created final figures for the paper.
- Drafted the manuscript. The manuscript was reviewed by Tobias Feuchtinger and the other co-authors.

1.2 Contribution to paper II

Leukemia
https://doi.org/10.1038/s41375-020-0793-1

ARTICLE

Acute lymphoblastic leukemia

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

Franziska Blaeschke[1] · Semjon Willier[1] · Dana Stenger[1] · Mareike Lepenies[1] · Martin A. Horstmann[2] · Gabriele Escherich[2] · Martin Zimmermann[3] · Francisca Rojas Ringeling[4] · Stefan Canzar[4] · Theresa Kaeuferle[1] · Meino Rohlfs[1] · Vera Binder[1] · Christoph Klein[1,4] · Tobias Feuchtinger[1]

I hereby confirm that I

- Drafted the proposal to the COALL study center and led the collaboration with the study center (together with Tobias Feuchtinger).
- Wrote funding grants (Bettina Braeu Stiftung, Gesellschaft fuer KinderKrebsForschung e.V.).
- Designed the experiments together with Tobias Feuchtinger.
- Established flow panels (including titration of antibodies and testing the panels). Some of the tests were performed by a technician from our lab and supervised by me.
- Established proliferation stain and stimulation protocol.
- Supervised Mareike Lepenies (who pipetted the experiments shown in figure 1/2) and helped with data analysis.
- Created Kaplan-Meier curves shown in figure 2 together with Mareike Lepenies and collaborated with Martin Zimmermann to receive data for figure 2H.
- Designed vectors for TIM-3 and CD200 overexpression.
- Chose and tested guide RNAs (gRNAs) for TIM-3 knockout (KO) and established CRISPR/Cas9-based TIM-3 KO (analysis on DNA and protein level).
- Designed experiments for figure 3 and performed data analysis. Pipetting was done by technicians from our lab and supervised by me.
- Coordinated RNA sequencing analysis shown in figure 4. Analysis was done by Stefan Canzar and Francisca Rojas Ringeling. RNA samples were frozen by Mareike Lepenies, library preparation and sequencing were done by Meino Rohlfs and Semjon Willier.
- Designed and analyzed CD200 experiments except for figure 4E which was provided by Semjon Willier. Pipetting for figure 4F-K was done by technicians in our lab under my supervision.
- Created final figures for the paper.
- Drafted the manuscript. The manuscript was reviewed by Tobias Feuchtinger and the other co-authors.

2. Introductory summary

2.1 Challenges for successful CAR T-cell therapy

The clinical success of autologous T cells genetically engineered to express a chimeric antigen receptor (CAR) against CD19 has revolutionized the treatment of certain cancer types and provides a curative treatment option for patients with otherwise dismal prognosis (1, 2). CD19 CAR T cells are usually produced by transducing T cells of the patient with a retro- or lentiviral construct encoding for an anti-CD19 single chain variable fragment (scFv) fused to costimulatory domains (i.e. CD28 or 4-1BB) and CD3zeta as part of the T-cell receptor (TCR) complex (3). Clinical studies led to FDA (U.S. Food and Drug Administration) and EMA (European Medicines Agency) approval of CD19 CAR T cells for the treatment of advanced B-cell malignancies in 2018 (4, 5). Nevertheless, despite >90% initial response rates in pediatric patients with relapsed/refractory B-cell precursor acute lymphoblastic leukemia (BCP-ALL), long-term remission rates dropped to about 50% in clinical studies due to relapse after CAR T-cell treatment (6, 7). Moreover, successful treatment with CAR T cells can be hampered by a variety of technical and biological challenges (figure 1): 1) Production failure of CAR T cells has been reported in up to 9% of the cases (8). 2) Antigen escape such as down-regulation of CD19 or outgrowth of CD19 splice variants can result in $CD19^-$ relapse (9, 10). 3) Toxicity of CD19 CAR T cells, in particular cytokine release syndrome (CRS) and neurotoxicity, can limit applicability (7). 4) Exhaustion, insufficient expansion and decreased persistence of CAR T cells due to both, T-cell intrinsic and extrinsic factors, can lead to $CD19^+$ relapse (11). 5) CAR T-cell therapy of solid tumors remains an unmet challenge due to lack of suitable antigens, tumor heterogeneity and difficulties in T-cell tumor invasion and persistence caused by the immune-suppressive tumor microenvironment (12).

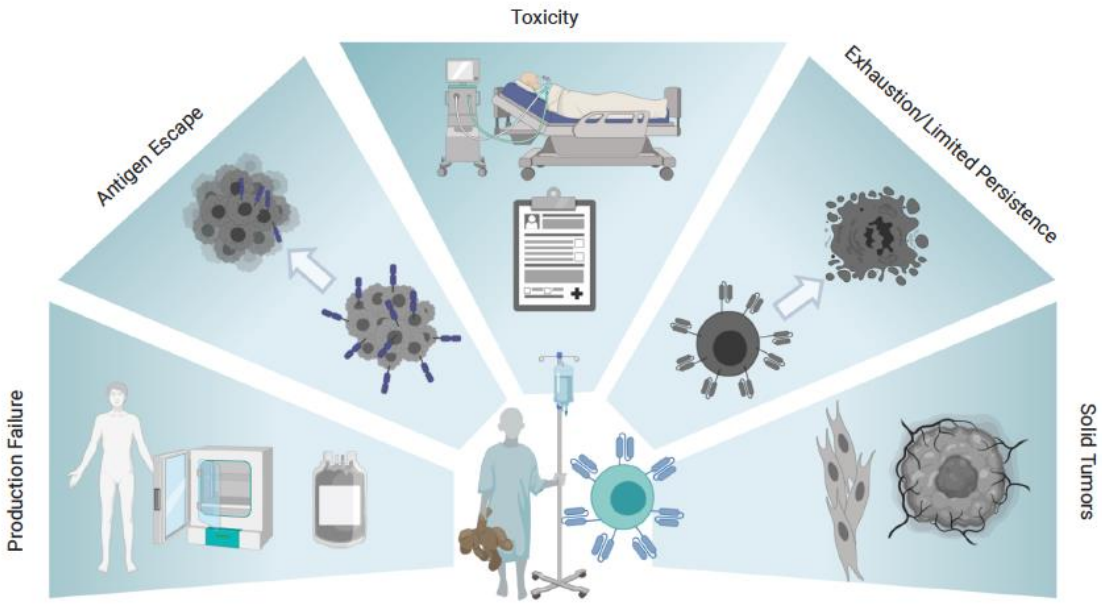


Figure 1: Challenges for CAR T-cell therapy. Created with BioRender.com.

This thesis addresses two of the major challenges. The first part will investigate how to produce a highly functional, standardized CAR T-cell product with favorable T-cell phenotype from peripheral blood of pediatric leukemia patients. The second part will evaluate the interaction of T cells with leukemic cells and introduces examples for how to improve current CAR T-cell therapies to break leukemia-mediated resistance.

2.2 Paper I: Automated CAR T-cell manufacturing for pediatric patients

2.2.1 Introduction

Manufacturing of CAR T cells is a time-consuming and complex procedure typically involving T-cell isolation, activation, lenti- or retroviral transduction and expansion (3). Before 2018, most of the protocols for large scale CAR T-cell production involved large culture flasks (i.e. G-Rex flasks), bioreactors or bags for expansion and a variety of open hands-on steps (13, 14). In order to standardize the production, Miltenyi Biotec had developed the CliniMACS Prodigy, a closed GMP-compatible (Good Manufacturing Practice) system for clinical scale manufacturing of CAR T cells (15, 16). The system had been tested and optimized using cell products from healthy donors but hadn't been tested with samples from pediatric leukemia patients yet. Thus, we aimed to test the process using 100-120 ml of peripheral blood from patients who were recently diagnosed with leukemia. Moreover, as studies suggested that CAR T-cell proliferation and persistence in the patients are crucial for a beneficial long-term outcome (17), we investigated the phenotype and expression level of exhaustion markers on the T cells and aimed to produce T cells with a favorable phenotype (18, 19) and high proliferative capacity upon repetitive antigen encounter.

2.2.2 Methods and Results

CD19 CAR T cells were successfully generated from peripheral blood of four pediatric patients using the CliniMACS Prodigy. T cells were isolated using CD4/CD8 microbeads, activated, lentivirally transduced with a 2nd generation CD19 CAR (4-1BB/CD3zeta) and expanded until day 12 after activation. CAR T-cell manufacturing at clinical scale was successful in 4/4 patients. The final product showed a mean transduction rate of 27% and a T-cell phenotype of mostly stem cell-like and central memory T cells. Leukemic blasts were not detectable in the final product, although 2/4 patients had >60% leukemic cells in their peripheral blood at timepoint of sample collection. Expression of exhaustion/co-inhibitory markers in the final product was low except for TIM-3 (T-cell immunoglobulin and mucin-domain containing-3). T cells from the final product showed high anti-leukemic functionality in vitro and strong proliferative capacity even after multiple antigen encounter. CAR T cells displayed a strong Th1 cytokine profile with increased secretion of IFN-γ (Interferon gamma), TNF-α (Tumor Necrosis Factor alpha), IL-2 (Interleukin-2) and GM-CSF

(Granulocyte-Macrophage Colony-Stimulating Factor). IL-6 production was low supporting previous reports that CAR T cells are not the major source of IL-6 in clinical CRS (20). CAR T-cell counts in the final product would have been sufficient to treat a 100 kg patient with a dose of up to 5e6 CAR T cells/kg body weight. In conclusion, CD19 CAR T-cell generation in a partly automated closed system for GMP-grade manufacturing was successful and yielded highly functional CAR T cells even from small pediatric patient samples.

2.2.3 Discussion

Automated and standardized manufacturing of CAR T-cell products at clinical scale has been challenging in the past and a variety of different production and expansion protocols have been utilized. Here, we verified that a partly automated closed system (15, 16) can be used even when starting with complex patient samples which consist of a variety of different cell types including leukemic blasts. Until now, CAR T-cell generation is still routinely done by using leukapheresis products from patients, a process which can be complicated due to the clinical situation of the patients. In this paper, we showed that peripheral blood as initial source can be used at least in older children. The presence of leukemic blasts in the leukapheresis or initial blood product can be concerning according to case reports about potential viral transduction of malignant B cells and subsequent $CD19^-$ relapse in patients (9). In our study, we weren't able to detect leukemic blasts at the end of the culture process, but as the staining was done with a conventional CD19 antibody and due to limited sensitivity of flow cytometry, we cannot fully exclude that this phenomenon can happen in patient samples with remaining blasts at timepoint of production start. Thus, optimizing the T-cell selection protocols for these patients is crucial. Besides remaining leukemic cells, patient samples can be challenging for a variety of other reasons such as non-homogenous starting material and T-cell exhaustion due to prior treatment. Nevertheless, our study has shown that CAR T-cell production using the CliniMACS Prodigy is feasible not only using samples from healthy donors but also with more complex patient samples. T cells in the final product showed high anti-leukemic functionality and proliferative potential as well as low expression of inhibitory molecules except for TIM-3. The CliniMACS Prodigy has since been used to produce CARs with a variety of specificities and clinical studies are ongoing (21, 22).

2.3 Paper II: The role of TIM-3 for anti-leukemic T-cell responses

2.3.1 Introduction

Although pediatric leukemia is considered a malignancy with low immunogenicity and low mutational load (23), earlier studies from our group had shown that leukemic cells can overexpress both, co-stimulatory and co-inhibitory immune checkpoint molecules (24). Moreover, bone marrow T cells of pediatric BCP-ALL patients can overexpress PD-1 (Programmed cell death protein 1) and TIM-3 and this expression can be increased by induction of a T-cell response

using the bispecific T-cell engager Blinatumomab (anti-CD3/CD19) (24). Thus, we were interested in more systematically studying the role of co-inhibitory immune checkpoints for prognosis and treatment of pediatric BCP-ALL. TIM-3 was in particular interesting because we had observed high TIM-3 expression on CAR T cells in paper I and TIM-3 (together with PD-1) was described to be overexpressed and associated with dismal prognosis in adult acute myeloid leukemia (AML) (25). TIM-3 has first been identified as an anti-inflammatory immune checkpoint on $CD4^+$ and $CD8^+$ T cells (26). Its known binding partners are HMGB1 (high mobility group box 1 protein), PtdSer (phosphatidylserine), CEACAM-1 (carcinoembryonic antigen-related cell adhesion molecule 1) and Galectin-9 (27-29). TIM-3 expression can be upregulated by inflammatory cytokines such as IL-2, -7, -12, -15, -21, -27 and TGF-β (Transforming Growth Factor beta) (30-33). TIM-3 is essential for immune tolerance in the context of auto-inflammatory disease and TIM-3 blockade by antibodies can lead to macrophage activation and experimental autoimmune encephalitis (26, 34, 35). In the context of chronic viral infections, TIM-3 was found to be expressed on exhausted and dysfunctional $CD8^+$ T cells (36, 37). In malignant disease, increased TIM-3 expression was observed on tumor-infiltrating lymphocytes in a variety of different solid tumors and was associated with T-cell dysfunction (38-40). Right before submission of this paper, presence of $PD\text{-}1^+/TIM\text{-}3^+$ $CD4^+$ T cells was shown to define a patient group with dismal prognosis in adult ALL (41), but TIM-3 expression had not been systematically studied in bone marrow samples from pediatric BCP-ALL before.

2.3.2 Methods and Results

We characterized leukemic cells and bone marrow T cells from 100 pediatric BCP-ALL patients and 13 healthy controls by flow cytometry and RNA sequencing. Follow-up data including relapse-free survival was available for all patient samples (median follow up of 7.8 years). Bone marrow was taken at timepoint of initial diagnosis before start of treatment. We found that CD4/CD8 ratio of bone marrow T cells was shifted toward a predominance of $CD4^+$ cells in the bone marrow of patients compared to healthy controls. Moreover, we found significantly higher percentages of terminally differentiated $CD62L^-/CD45RO^-$ T cells in the bone marrow of patients. PD-1 and TIM-3 were not significantly upregulated on T cells of BCP-ALL patients, but patients with future relapse of leukemia had a significantly higher proportion of $TIM\text{-}3^+/CD4^+$ T cells in their bone marrow at timepoint of initial diagnosis compared to patients who will survive in complete remission. We thus evaluated the predictive value of TIM-3 expression (with or without co-expression of PD-1) for relapse-free survival of BCP-ALL patients. We found that TIM-3 expression alone (as well as in combination with PD-1 expression, but not PD-1 expression alone) can predict future ALL relapse with a hazard ratio of 7.1. We next confirmed that contact with leukemic blasts and induction of a T-cell response by Blinatumomab can induce TIM-3 expression on primary human T cells. We used CRISPR/Cas9 technology to knock out (KO) TIM-3 expression in primary human T cells and found that TIM-3 KO increases activation and proliferation in T cells while viral overexpression of TIM-3 leads to decreased proliferative potential of T cells in response to leukemic blasts. RNA sequencing of patients' bone marrow samples revealed that – while known inducers and ligands of TIM-3 were not differentially expressed

between TIM-3 high vs low bone marrow samples – the co-inhibitory immune checkpoint CD200 was significantly upregulated in leukemia cells derived from TIM-3 overexpressing bone marrow samples (multiple testing correction performed for the 21 immune-regulatory genes tested in this analysis). We indeed found that CD200 is highly overexpressed on leukemic cells compared to physiological precursors and that CD200 expression on leukemic cells can decrease activation potential of anti-leukemic T cells. Moreover, we observed a subtle but statistically significant overexpression of TIM-3 on $CD4^+$ T cells induced by contact with $CD200^+$ leukemic cells both on transcriptional and protein expression level.

2.3.3 Discussion

Although pediatric leukemia is considered a malignancy of low immunogenicity, we found that T cells are present in the bone marrow of pediatric BCP-ALL patients and show a more terminally differentiated phenotype compared to healthy controls. Further studies have to elucidate whether the altered phenotype would have been detectable prior to diagnosis and could represent a risk factor for developing a hematologic malignancy, or whether leukemia itself induced terminal differentiation in T cells. Moreover, we found that TIM-3 expression on $CD4^+$ bone marrow T cells of pediatric patients can reliably predict leukemia relapse. Despite slightly lower statistical significance of prediction based on TIM-3 expression compared to the conventionally used prediction marker MRD (minimal residual disease), the hazard ratio was comparable (7.12-fold increased risk for TIM-3 high patients vs 7.98-fold increased risk defined by MRD quantification in this cohort). In our study, RNA sequencing analysis suggested that TIM-3 expression in bone marrow T cells was not upregulated by known inducers but was associated with elevated CD200 expression on leukemic cells. CD200 overexpression was observed in a variety of different solid tumors as well as in leukemia (42-45). It's known interaction partner, CD200 receptor (CD200R), is expressed on antigen presenting cells (APCs) and T cells and binding of CD200 can lead to decreased T-cell function (46). More mechanistic insight is needed regarding whether TIM-3 can directly be induced by CD200 or if there is an indirect mechanism leading to the subtle TIM-3 overexpression we observed in this study. Moreover, it is unclear whether CD200 can only increase TIM-3 expression (directly or indirectly) or if it can also mediate TIM-3 function. Nevertheless, we confirmed that TIM-3 overexpression decreases T-cell activation whereas TIM-3 KO improves activation and proliferation capacity of anti-leukemic T cells. In conclusion, the data showed that BCP-ALL is associated with late T-cell differentiation states and suggested that the TIM-3 axis constitutes a mechanism of immune dysregulation in BCP-ALL. This finding is not only important to better understand the potential interaction of leukemic cells and bone marrow T cells but can also inform future therapeutic approaches. It underlines the rationale for testing e.g. TIM-3 KO CD19 CAR T cells, checkpoint blockade with anti-TIM-3 or administration of T cells with e.g. TIM-3/CD28 fusion receptors which are meant to turn TIM-3-mediated inhibition into T-cell activation through CD28.

2.4 Conclusion and Outlook

Clinical treatment of pediatric BCP-ALL involves chemotherapy and – in case of high-risk leukemia or relapse – treatment with the bi-specific T-cell engager Blinatumomab and hematopoietic stem-cell transplantation. CD19 CAR T cells are currently approved for refractory disease or after multiple relapses. Administration of CD19 CAR T cells as an earlier line in BCP-ALL therapy is investigated in clinical trials. Despite high initial response rates after treatment with CD19 CARs, long-term event-free survival decreases to about 50% in pediatric BCP-ALL patients (6, 7). Clinical trials are investigating combination therapies of CAR T cells with checkpoint blockade, but checkpoint blockade alone so far had only limited success in malignancies with low immunogenicity/mutational load such as pediatric BCP-ALL (47, 48). To improve current treatment of pediatric BCP-ALL, it is not only important to optimize and standardize production protocols (as shown in paper I), but also to better understand the interaction of leukemic cells with bone marrow T cells (as shown in paper II). Understanding that, although dealing with a low-immunogenic malignancy, immune checkpoint axes can play a role for prognosis and treatment of pediatric leukemia is crucial for hypothesis-driven development of new immunotherapeutic approaches. In currently unpublished data, we pursued the therapeutic role of immune checkpoints and found that CD19 CAR T cells with TIM-3 KO or with additional overexpression of TIM-3/CD28 or PD-1/CD28 fusion receptors (49) are better able to control leukemia than conventional CAR T-cell approaches. We hope that a better insight into the interplay of therapeutic and physiologic T cells with leukemic cells can inform future translational research projects and ultimately lead to better CAR T-cell therapies.

3. Paper I

Material from: Blaeschke et al., "Induction of a central memory and stem cell memory phenotype in functionally active CD4+ and CD8+ CAR T cells produced in an automated good manufacturing practice system for the treatment of CD19+ acute lymphoblastic leukemia", Cancer Immunology, Immunotherapy, published 2018, Springer Nature

Cancer Immunology, Immunotherapy (2018) 67:1053–1066
https://doi.org/10.1007/s00262-018-2155-7

ORIGINAL ARTICLE

Induction of a central memory and stem cell memory phenotype in functionally active CD4+ and CD8+ CAR T cells produced in an automated good manufacturing practice system for the treatment of CD19+ acute lymphoblastic leukemia

Franziska Blaeschke[1] · Dana Stenger[1] · Theresa Kaeuferle[1] · Semjon Willier[1] · Ramin Lotfi[2,3] · Andrew Didier Kaiser[4] · Mario Assenmacher[4] · Michaela Döring[1,5] · Judith Feucht[5,6] · Tobias Feuchtinger[1]

Received: 14 August 2017 / Accepted: 24 March 2018 / Published online: 31 March 2018

Abstract
Relapsed/refractory B-precursor acute lymphoblastic leukemia (pre-B ALL) remains a major therapeutic challenge. Chimeric antigen receptor (CAR) T cells are promising treatment options. Central memory T cells (Tcm) and stem cell-like memory T cells (Tscm) are known to promote sustained proliferation and persistence after T-cell therapy, constituting essential preconditions for treatment efficacy. Therefore, we set up a protocol for anti-CD19 CAR T-cell generation aiming at high Tcm/Tscm numbers. 100 ml peripheral blood from pediatric pre-B ALL patients was processed including CD4+/CD8+-separation, T-cell activation with modified anti-CD3/-CD28 reagents and transduction with a 4-1BB-based second generation CAR lentiviral vector. The process was performed on a closed, automated device requiring additional manual/open steps under clean room conditions. The clinical situation of these critically ill and refractory patients with leukemia leads to inconsistent cellular compositions at start of the procedure including high blast counts and low T-cell numbers with exhausted phenotype. Nevertheless, a robust T-cell product was achieved (mean CD4+ = 50%, CD8+ = 39%, transduction = 27%, Tcm = 50%, Tscm = 46%). Strong proliferative potential (up to > 100-fold), specific cytotoxicity and low expression of co-inhibitory molecules were documented. CAR T cells significantly released TH1 cytokines IFN-γ, TNF-α and IL-2 upon target-recognition. In conclusion, partly automated GMP-generation of CAR T cells from critically small blood samples was feasible with a new stimulation protocol that leads to high functionality and expansion potential, balanced CD4/CD8 ratios and a conversion to a Tcm/Tscm phenotype.

Poster presentation at 58th ASH Annual Meeting and Exposition (American Society of Hematology), December 3–6 2016, San Diego, USA [1].

Poster presentation at Cellular Therapy, International Symposium Erlangen, March 16–17 2017, Erlangen, Germany [2].

Oral presentation at 43rd Annual Meeting of the European Society for Blood and Marrow Transplantation (EBMT), March 26–29 2017, Marseille, France [3].

Oral presentation at Annual Meeting of Paediatrische Arbeitsgemeinschaft für Stammzelltransplantation and Zelltherapie (PAS&ZT), September 14–15 2017, Hamburg, Germany [4].

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00262-018-2155-7) contains supplementary material, which is available to authorized users.

Extended author information available on the last page of the article

1054 Cancer Immunology, Immunotherapy (2018) 67:1053–1066

Keywords CAR T cells · GMP production · Tscm/cm · Pediatric ALL

Abbreviations

6-MP	6-Mercaptopurine
6-TG	6-Thioguanine
ARA-C	Cytarabine
B-NHL	B-cell non-Hodgkin's lymphoma
cALL	Common acute lymphoblastic leukemia
CNS	Central nervous system
DNR	Daunorubicin
FMO	Fluorescence minus one
HIDAC	High-dose cytarabine
HSA	Human serum albumin
MOI	Multiplicity of infection
MTX	Methotrexate
PB	Peripheral blood
PEG-ASP	Pegylated asparaginase
Pre-B ALL	B-precursor acute lymphoblastic leukemia
Tcm	Central memory T cells
TCT	T-cell transduction
Teff	Effector T cells
Tem	Effector memory T cells
Tscm	Stem cell-like memory T cells
VCN	Vector copy number
VCR	Vincristine
VP16	Etoposide

Introduction

Treatment with genetically modified T cells has the potential to induce sustained clinical remissions even in patients with relapsed or primary refractory disease. In particular, T cells genetically directed against the B-cell lineage antigen CD19 are currently evaluated in more than 40 clinical phase I/II trials. Preliminary results underline their enormous potential in B-lineage malignancies. Especially in pediatric pre-B ALL, response rates of up to 95% have been shown in refractory, heavily pre-treated patients [5–10]. Those T cells are typically generated by retro- or lentiviral transduction of patient-derived autologous T cells with a vector encoding for a chimeric antigen receptor (CAR) consisting of the anti-CD19 single-chain variable fragment (ScFv) fused to CD3ζ as a domain of the T-cell receptor (TCR) complex [11]. However, low cell number in pediatric samples, exhausted T cells under treatment protocols and the presence of leukemic cells in the starting fraction are challenges for stable production of functionally active CAR T-cell products.

Although the vast majority of pediatric ALL patients respond to second generation anti-CD19 CAR T-cell treatment, there are patients not responding or relapsing after T-cell therapy. Leukemia relapse or initial non-response is typically caused by early loss or non-engraftment of anti-CD19 CAR T cells [6, 12, 13]. Although associated with B-cell aplasia, Maude et al. underlined the impact of prolonged anti-CD19 CAR T-cell persistence through sustained in vivo proliferation on long-term outcome of pediatric ALL patients [12]. Thus, we aimed at generating CAR T cells with a T-cell phenotype in favor of high proliferative potential. Central memory (Tcm) or stem cell-like memory (Tscm) T cells are known to be best candidates for a sustained in vivo response after adoptive T-cell transfer as they display good effector functions while maintaining their high proliferative capacity [14–16]. Moreover, leukemic cells are known to up-regulate inhibitory molecules such as PD-L1 to circumvent T-cell mediated TH1 attack [17]. Generation of anti-CD19 CAR T cells with low sensitivity to inhibitory checkpoint signals thus might play a crucial role for sustained remissions after T-cell therapy [18].

Although CAR T-cell generation is well established in many laboratories, it is a complex procedure originally involving many hands-on/open steps [19]. To simplify and to facilitate standardization of the process, a protocol for partly automated large scale CAR T-cell manufacturing was established on a closed, GMP-compatible device requiring only few open hands-on steps [20]. As the activity protocol has a modular structure, it can be adapted for various conditions such as different starting cell counts, varied CD4/8 proportion, feeding or washing steps and final formulation. Here, we demonstrate feasibility of partly automated, GMP-compatible clinical-scale manufacturing of anti-CD19 CAR T cells on a closed device even from very small pediatric patients' samples.

Materials and methods

Patients

Patients' characteristics are shown in Table 1.

Peripheral blood mononuclear cell (PBMC) generation

PBMCs were generated from 100 to 120 ml PB of pediatric ALL patients by density gradient centrifugation (Biocoll, Biochrom, Berlin, Germany). Cells were frozen in 5% human serum albumin (HSA) (Biotest, Dreieich, Germany) containing 10% DMSO (Sigma, Taufkirchen, Germany).

Table 1 Characteristics of patients and anti-CD19 CAR T-cell products

	Patient CAR001	Patient CAR002	Patient CAR003	Patient CAR004
Patient-related information				
Gender	Female	Female	Female	Female
Age at time-point of blood sampling	18	15	14	18
Diagnosis	cALL	cALL	cALL	cALL relapse
Age at diagnosis	17	15	14	18 Initial diagnosis: 13
CNS state	Negative	Negative	Negative	Negative
Cytogenetics	TEL deletion 12p13 MLL deletion 11q23 ABL trisomy 9q34	BCR-ABL fusion	My^+	High-hyperdiploid karyotype
Treatment protocol	CoALL Protocol 08/09	EsPhALL-CoALL Protocol 08/09	CoALL Protocol 08/09	ALL REZ BFM Protocol
Day of blood sampling according to protocol	Before start of day 92 treatment	Before treatment	Before treatment	After first R2 block
Systemic chemotherapy received for current diagnosis until date of blood sampling	Prednisolone DNR, VCR, HIDAC, PEG-ASP, MTX, VP16, ARA-C, 6-MP, 6-TG	None	None	Dexamethasone VCR, MTC, PEG-ASP, ARA-C IDA, CPM, 6-TG, 6-MP, VDS, IFO, DNR
Disease activity at time-point of blood sampling	MRD negative	71% blasts in PB	64% blasts in PB	MRD negative
Process-related information				
Volume of blood sample (ml)	120	100	100	100
T-cell count after CD4/8 separation	1.74×10^7	4.89×10^7	3.86×10^7	9.36×10^6
T-cell count of final product	3.35×10^9	1.53×10^9	4.87×10^8	8.87×10^8
T-cell transduction rate (%)	40.4	19.6	19.3	28.5
Absolute number of CAR^+ T cells in final product	1.35×10^9	3.00×10^8	9.40×10^7	2.53×10^8

cALL common acute lymphoblastic leukemia, *CNS* central nervous system, *DNR* daunorubicin, *VCR* vincristine, *HIDAC* high-dose cytarabine, *PEG-ASP* pegylated asparaginase, *MTX* methotrexate, *VP16* etoposide, *ARA-C* cytarabine, *6-MP* 6-mercaptopurine, *6-TG* 6-thioguanine, *MRD* minimal residual disease, *PB* peripheral blood

Flow cytometry

Cells were stained for CD3-FITC, CD8-APC-Vio 770, CD4-VioGreen, CD69-PE-Vio 770, CD25-PE, CD137-APC, CD56-PE, CD14-PerCP, CD19-PE-Vio 770, 7-AAD, CD45RO-PE-Vio 770, CD62L-VioBlue, CD95-APC, CD95-PE, OX-40-PE-Vio 770 (Miltenyi Biotec), CD4-BV650, CTLA-4-APC, CD3-BUV395 (Becton, Dickinson and Company (BD), Franklin Lakes, NJ, USA), CD8-PE/Cy7, CD137-BV421, CD56-BV421, 2B4-APC, BTLA-BV421, CD10-APC, TIM-3-BV785, PD-1-BV421, CTLA-4-PE/Cy7, PD-L1-BV421, CD8-PerCP/Cy5.5 (Biolegend, San Diego, CA, USA), VISTA-APC (R&D Systems, Minneapolis, MN, USA), TIGIT-APC, LAG-3-PE, Fixable Viability Dye eFluor 780 (eBioscience/Thermo Fisher Scientific, Waltham, MA, USA). Transduction rate was evaluated by staining with 5 ng/ml Biotin-Protein L (GenScript, Piscataway, NJ, USA) and anti-Biotin-APC or -PE (Miltenyi Biotec). Intracellular stains for IFN-γ-PE (BD) and TNF-α-PacificBlue (Biolegend) were performed using Fix and Perm Cell Permeabilization Kit (Thermo Fisher Scientific). Flow cytometric measurements were performed on a MACSQuant Analyzer 10 (Miltenyi Biotec) or BD LSRFortessa (BD).

T-cell transduction process

Material of the CliniMACS process was from Miltenyi Biotec, Bergisch Gladbach, Germany, unless otherwise stated. The CliniMACS Prodigy is an automated, closed system constructed for large-scale generation of immunotherapeutic T-cell products in a GMP-accredited way. It consists of 24 valves into which a sterile, closed tubing set is inserted without direct contact to the device. All parts intended to come into contact with the cellular product are sterile and

single-use materials. The tubing set contains a single-use culture chamber which is fixed into the CentriCult Unit of the CliniMACS Prodigy where optimal culturing conditions of 37 °C/5% CO_2 are achieved and the cell product can be centrifuged and shaken during the process.

After written informed consent from patients/their parents, 100–120 ml PB were taken from the patients' central venous access (Hickman catheter/port), PBMCs were generated and frozen. The frozen product was thawed in prewarmed TexMACS GMP medium and immediately transferred to the CliniMACS Prodigy on day 0 by sterile tube welding without additional washing steps. Tubing set CliniMACS Prodigy TS520 was used together with TCT software version 1.0. CliniMACS PBS/EDTA supplemented with 0.5% HSA (Grifols, Barcelona, Spain) served as process buffer for separation with CliniMACS CD4/CD8 reagent. Cell culture was set-up after selection for $CD4^+$/$CD8^+$ cells by modular programming the activity matrix of the process (Supplementary Table 1). Culture medium consisted of TexMACS GMP medium, 3% human AB serum (Institute for Clinical Transfusion Medicine, Ulm, Germany), 12.5 ng/ml MACS GMP Recombinant Human IL-7 and IL-15. Medium bag was exchanged for a fresh one on day 6 containing only interleukins, no serum was added. For activation, one vial TransAct T Cell Reagent-Large Scale, human was used according to manufacturer's recommendations (working dilution of 17.5 of the vial). Activation reagent was washed out together with the lentivirus on day 2 of the process, no magnetic bead removal step was required. One day after activation, T-cell transduction was performed with a second generation CAR lentiviral vector encoding for anti-CD19 single-chain variable fragment (scFv), 4-1BB (CD137) co-stimulatory domain and CD3ζ chain. ScFv sequence was derived from mouse hybridoma FMC-63 (AA 1-267, GenBank ID: HM852952.1), $(GGGGS)_3$ was used as linker domain as described by Schneider et al. [21]. Lentivirus was thawed at room temperature and diluted with TexMACS GMP medium to a final volume of 10 ml. The suspension was transferred to a 150 ml Transfer Bag which was welded to the tubing set. Multiplicity of infection (MOI) rates are shown in Supplementary Table 2. Activation reagent/virus were washed out on day 2. On day 12, cells were harvested in a final volume of 100 ml TexMACS GMP medium. Cells were frozen in 5% human serum albumin (Grifols, Barcelona, Spain) containing 10% DMSO (Sigma, Taufkirchen, Germany). Flow cytometric/functionality analyses were either performed immediately after harvest or from frozen cells which were thawed in warm TexMACS GMP medium. Untransduced MOCK controls were taken from reapplication bag after separation for CD4/8. MOCK cells were cultured in 48-well plates (Corning, Corning, New York, USA) and treated similar to large scale runs except for transduction. Samples were taken whenever needed using the automated sterile sampling system. In case three sampling pouches were not sufficient, an additional Triple Sampling Adapter was welded to the tubing set. Sterile tube welding was performed with an SC-201AH TSCD Sterile Tube Welder (Terumo).

PBMC isolation, buffer/medium/virus preparation and cryopreservation required grade A clean room conditions (open/manual steps). Spiking and sealing procedures are indicated in Fig. 1. All other steps were performed in the closed device (CliniMACS Prodigy placed in clean room grade B or C).

Small scale runs to compare CD4/CD8-separated cells with PBMCs were performed similar to the large scale protocol. The amount of virus for transduction was calculated based on the T-cell count. 1×10^6 T cells were transduced with 2×10^6 particles.

Cytotoxicity assay

Target cells were labeled according to CellTrace Violet Cell Proliferation Kit (Thermo Fisher Scientific) and co-cultured with CAR T cells for 24 h. Effector-cell count was calculated based on transduction rate (CAR^+ cells) which was analyzed by flow-cytometric staining for Protein-L. Absolute count of remaining CellTrace Violet-positive cells was calculated with the MACSQuant Analyzer 10 (Miltenyi Biotec) and set into relation to the count of CellTrace Violet-positive cells of control wells (target cells only).

Proliferation assay

T cells were labeled according to CellTrace Violet Cell Proliferation Kit (Thermo Fisher Scientific) and co-cultured with Jeko cells at an E:T ratio of 5:1 (2×10^4 target cells). Effector-cell count was calculated based on transduction rate (CAR^+ cells) which was analyzed by Protein-L stain. T cells were re-stimulated every 24 h with 2×10^4 target cells. Cell proliferation was measured on a MACSQuant Analyzer 10 (Miltenyi Biotec) before stimulation with targets and 24 h after each re-stimulation.

MACSPlex assay

CAR T cells were co-cultured with target cells at an E:T ratio of 1:2. Effector-cell count was calculated based on transduction rate (CAR^+ cells) analyzed by flow-cytometric staining for Protein-L. Supernatant of co-cultures was harvested after 24 h and frozen at − 20 °C. For analysis, supernatant was thawed and diluted 1:5 with buffer supplied with the MACSPlex Cytokine 12 kit (Miltenyi Biotec). Flow-cytometric measurement was performed according to supplier's information.

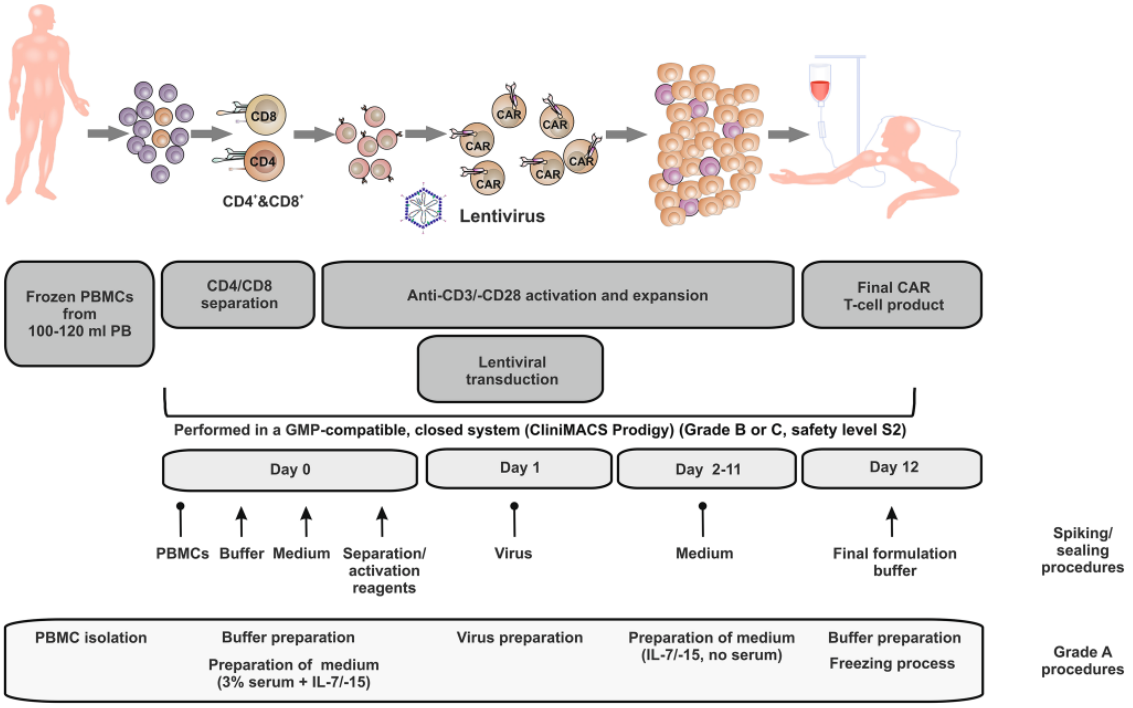


Fig. 1 Schematic representation of T-cell transduction (TCT) process on the CliniMACS Prodigy: PBMCs from pediatric ALL patients were thawed and immediately transferred to the device on day 0. After automated CD4/8 separation and CD3/28 activation, transduction with the lentiviral anti-CD19 CAR vector was performed on day 1. CAR T cells were expanded for 12 days until final CAR T-cell product was harvested from the device. For the clinical setting, the CliniMACS Prodigy is currently located in GMP grade B or C. Open steps (GMP grade A), spiking (↑) and sealing (🔒) procedures are indicated in the lower part of the figure. Cells are cultured in safety level S2. Due to multiple washing and dilution steps, the final product is downscaled to safety level S1. *PBMC* peripheral blood mononuclear cells, *PB* peripheral blood, *CAR* chimeric antigen receptor, *GMP* good manufacturing practice

qPCR

To determine lentiviral copy numbers, real-time qPCR was used. Amplification of vector-specific sequences (gag) was compared to host-specific sequences (PTBP2) using Taqman Fast Advanced Master Mix (Thermo Fisher Scientific) and the BIO-RAD CFX96 Touch Real-Time PCR Detection System.

Statistics

Statistical analyses were performed using Graphpad Prism 7.

Results

Anti-CD19 CAR T-cell generation using small peripheral blood samples from pediatric ALL patients

Autologous anti-CD19 chimeric antigen receptor (CAR) T-cell products were generated on the CliniMACS Prodigy from 100 to 120 ml peripheral blood of four different pediatric patients. The CliniMACS Prodigy is an automated, closed system constructed for large-scale generation of T-cell products in a GMP-accredited way (Fig. 1). Patients' characteristics are shown in Table 1. Patients' age ranged from 14 to 18 years (mean 16.25 years). All four patients were female and suffered from B-precursor acute lymphoblastic leukemia (common ALL, cALL), either after relapse ($n=1$) or after primary diagnosis ($n=3$). None of the patients had central nervous system (CNS) disease. Patients were either pre-treated with standard chemotherapy according to CoALL 08/09 protocol ($n=1$) or ALL REZ BFM

2012 protocol ($n=1$), respectively, or evaluated at time-point of diagnosis, and thus prior to chemotherapeutic treatment ($n=2$). Blood of pre-treated patients was taken between two cycles of chemotherapy after lymphocyte counts had resolved to > 500/µl. Blood was not taken during treatment with corticosteroids to avoid inhibitory effects on the T cells. At time-point of sampling, minimal residual disease of two patients was lower than detection limit by polymerase chain reaction (PCR) in the bone marrow, whereas the other two patients suffered from morphological disease (71 or 64% blasts in peripheral blood, respectively).

Initial T-cell count of the starting fraction of the four patients ranged between 9.36×10^6 and 4.89×10^7 T cells (mean 2.86×10^7) after CD4/8 separation. Although the process is optimized for an initial cell product of 1×10^8 T cells, generation of sufficient amounts of anti-CD19 CAR T cells was feasible from these very low T-cell numbers (Table 1). After 12 days of expansion on the CliniMACS Prodigy, a mean of 4.99×10^8 CAR^+ T cells (range 9.4×10^7 to 1.35×10^9) was achieved, suitable for the treatment of a 100 kg patient with a dose of up to 4.99×10^6 CAR^+ T cells/kg body weight.

Anti-CD19 CAR T cells derived from pediatric patients show high expansion potential and transduction rates

T cells were activated on day 0 of the process with a new anti-CD3/-CD28-based activation reagent (TransAct T Cell Reagent-Large Scale, human) and were cultured in the presence of IL-7 and IL-15. Microscope pictures of the integrated microscope showed clustering of T cells 24 and 48 h after activation indicating efficient T-cell activation (Fig. 2a). When analyzed by flow cytometry 48 h after activation, T cells expressed activation markers CD69, CD25 and CD137 (Fig. 2b). Cell count/viability were evaluated on day 0, 2, 5, (6), 7, 9 and at the end of the process by sterile sampling from the device. Mean expansion rate of lymphocytes within the culture was 48-fold (range 14.7–102.4) although two out of four patients had received corticosteroids in previous chemotherapy cycles (Fig. 2c). A slight decrease in cell count was detectable on day 2 of the process possibly due to cell death caused by thawing, activation and transduction. Viability did not fall below a mean of 95.8% during the process (Fig. 2d). For CAR002, cell count decreased below detection threshold on day 2 of the process and survival of T cells was confirmed by re-concentration through additional centrifugation of the material provided in the sampling pouch. Microscope pictures of the culture chamber showed massive cell clumps associated with the destruction of leukemic cells. Viability and cell count resumed within the next three days of culture. A trend towards decreased T-cell expansion in the presence of blasts was observed (Fig. 2e).

Lentiviral transduction was performed on day 1 after sterile welding the virus-containing bag to the tubing set of the device. A viral concentration of 2×10^8 particles per 100 ml was used, irrespective of T-cell counts. Frequency of CAR^+ T cells was analyzed by flow-cytometric stain for Biotin-protein L and detected by anti-Biotin-APC or -PE (Fig. 3a). Mean T-cell transduction rate at the end of the process was 26.95% (Fig. 3b). Multiplicity of infection (MOI) and corresponding transduction rates are shown in Supplementary Table 2. Transduced cells consisted of mainly CD4 and CD8 T cells, although a small amount of $CD3^+/CD56^+$ NKT cells was also transduced. The cellular composition among CAR^+ cells showed a slight but not statistically significant predominance of $CD4^+$ T cells followed by $CD8^+$ T cells (Fig. 3c). No B cells or leukemic blasts were transduced. Transduced NKT cells displayed a predominant $CD3^+/CD8^+$ NKT-cell phenotype (Supplementary Figure 1). The number of viral copies (vector copy number) determined by real-time PCR of genomic DNA did not exceed 2.3 per cell (mean 1.72), and thus met the requirements of authorities to decrease the potential risk of insertional mutagenesis.

Balanced CD4/CD8 ratio with predominance of Tcm and Tscm T-cell phenotype and low expression of inhibitory checkpoint molecules

Cellular composition was evaluated by flow cytometry prior to and after CD4/CD8 separation on day 0 as well as on day 5 and at the end of the process (Fig. 4a). Gating strategies/exemplary plots are shown in Supplementary Figure 4. The patient-derived initial product showed a huge variety in cellular composition including high blast and monocyte counts and a mean of only 22% $CD4^+$ T cells (range 2.74–39.5%) and 16.73% $CD8^+$ T cells (range 1.7–28%). Although CD4/8 separation led to enrichment of both cell types (mean of 45.25% for $CD4^+$ cells and 20.84% for $CD8^+$ cells), monocytes and blasts were still present in the culture after separation. Already after five days of IL-7/-15-based expansion, no B cells, leukemic blasts or monocytes were detectable anymore. The final product contained a robust cellular composition with a mean of 50.3% $CD4^+$ and 38.7% $CD8^+$ cells. A mean of 10.2% of the cells were NKT cells. The T-cell phenotype of the initial and the final product was analyzed by flow cytometric stain for CD62L, CD45RO and CD95 (Fig. 4b). Gating strategy is shown in Supplementary Figure 5. Whereas the initial product showed a broad variety and rather exhausted phenotype, the final CAR T-cell product consisted mostly of Tcm and Tscm cells which are known to have excellent proliferative potential and functionality in vivo. No increased expression of exhaustion/senescence markers CD57, 2B4, PD-1 and LAG-3

Cancer Immunology, Immunotherapy (2018) 67:1053–1066

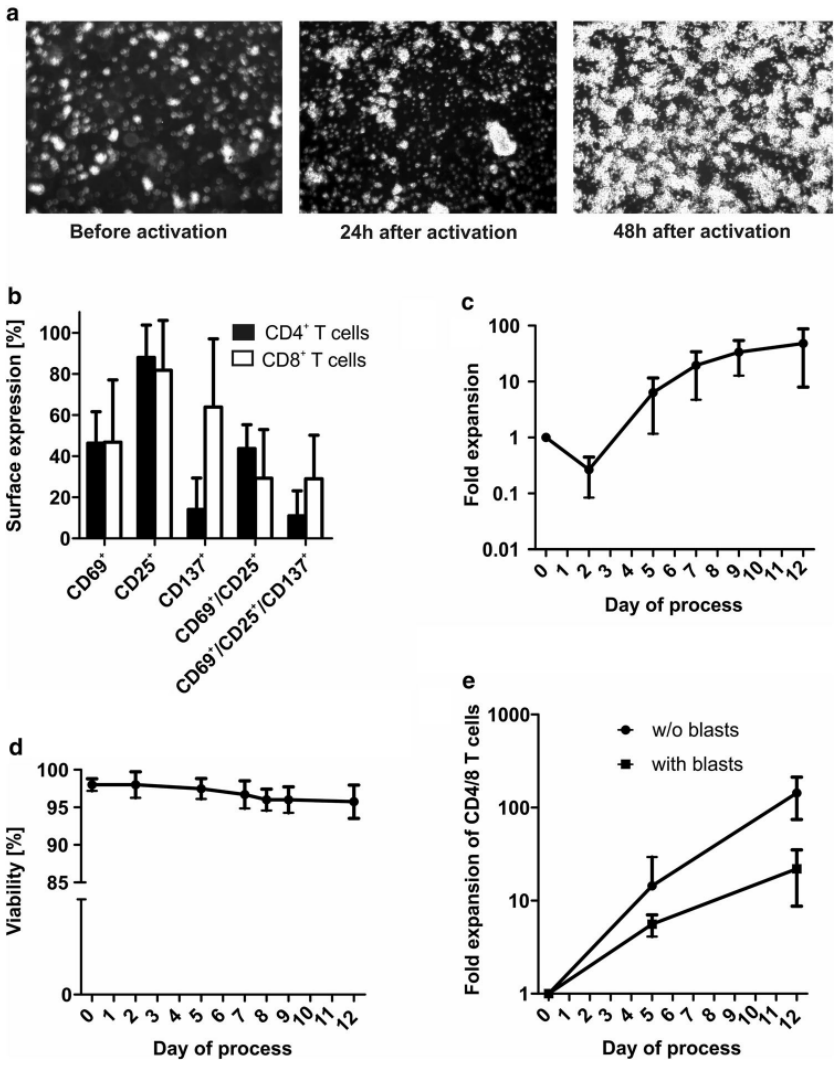


Fig. 2 Activation, expansion and viability during TCT process. **a** Exemplary microscope pictures of the T-cell culture before, 24 and 48 h after activation with CD3/CD28-based TransAct T Cell Reagent showed clustering of T cells as a sign of T cell activation (400-fold magnification, taken with microscope included in the CliniMACS Prodigy). **b** T cells expressed various activation markers 48 h after CD3/CD28 activation ($n=2$). **c** A mean expansion rate of 47.9-fold was achieved during the process (overall cell count in culture chamber, $n=4$). **d** Mean viability was higher than 95% throughout the protocol ($n=4$). For CAR002, cell count decreased below detection threshold on day 2 of the process and survival of T cells was only confirmed by re-concentration through additional centrifugation of the material provided in the sampling pouch. **e** A trend towards decreased T-cell expansion in the presence of blasts was observed (gated on $CD4^+/CD8^+$ T cells, $n=2$ for each group)

was measurable on the CAR^+ or CAR^- T cells of the final product (Fig. 4c). Only isolated expression of TIM-3 was found on CAR^- and CAR^+ T cells (data not shown). Low sensitivity to inhibitory signals caused by leukemic cells was detected by low surface expression of inhibitory checkpoint molecules BTLA, PD-1, VISTA, TIGIT and CTLA-4 (Fig. 4d). A significantly different expression between CAR^+ and CAR^- cells was not detectable.

TH1 driven specific functionality of anti-CD19 CAR T cells

After co-culture with a $CD19^+$ cell line, anti-CD19 CAR T cells dose-dependently killed up to 80% of the target cells at an E:T ratio of 5:1 (Fig. 5a). Anti-CD19 CAR T cells co-cultured with a $CD19^-$ cell line served as control. CAR T cells proliferated up to 20-fold when co-cultured with a $CD19^+$ cell line even after several re-stimulations (Fig. 5b, c). In a second step, cytokine release was measured in the co-culture supernatant by flow cytometry (Fig. 6a). After contact to a $CD19^+$ cell line, CAR T cells were able to secrete GM-CSF, IFN-γ, IL-2, and TNF-α, indicating a strong TH1 response of the CAR T cells. IL-5 and IL-10 were also secreted significantly higher compared to negative controls but stayed at very low concentrations (mean of 63.36 pg/ml for IL-5, mean of 138.79 pg/ml for IL-10). Interestingly, although many clinical studies detect high amounts of IL-6 after anti-CD19 CAR T-cell infusion, a

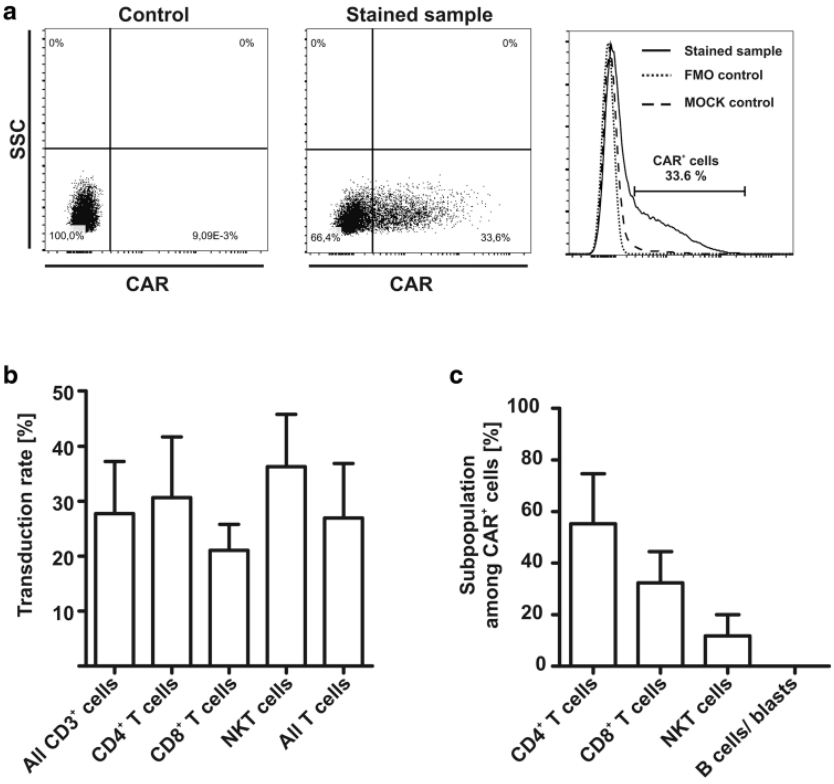


Fig. 3 T-cell transduction with anti-CD19 CAR lentiviral vector. **a** Transduction rate was analyzed by flow cytometric stain for Biotin-Protein L and secondary stain for anti-Biotin-PE or -APC. Cells stained only with the secondary antibody (FMO) or untransduced T cells were used as controls. **b** Transduction rate among different cell subsets was analyzed by flow cytometry and showed no significant difference between the subsets. Mean T-cell transduction rate was 26.95% ($n=4$). Two-tailed unpaired *t* test was performed to determine statistical significance. **c** Cellular composition of CAR^+ cells was analyzed by flow cytometry ($n=4$). Transduced T cells consisted of $CD4^+$ T cells, $CD8^+$ T cells and NKT cells. No transduced B cells or blasts were detected. Two-tailed unpaired *t* test was performed to determine statistical significance. FMO: fluorescence minus one

significant but only very low production of IL-6 was detectable after co-culture with target cell lines (mean 72.07 pg/ml). PMA/Ionomycin-stimulated T cells served as positive, CAR T cells co-cultured with a $CD19^-$ cell line and untransduced T cells co-cultured with a $CD19^+$ cell line served as negative controls. For further analysis, we co-cultured CAR T cells with autologous target cells using the negative fraction derived from CD4/8 separation on day 0 of the process (termed here "autologous B cells/blasts"). The negative fraction consisted of mainly $CD19^+$ B cells and $CD19^+$/$CD10^+$ leukemic blasts (Supplementary Figure 2). When co-cultured with these autologous B cells/blasts, CAR T cells showed a similar TH1-based cytokine release profile (Fig. 6b). Interestingly, compared to experiments with target cell lines, the supernatant of those co-cultures showed a higher amount of IL-6 (mean of 1702.67 pg/ml) supporting recent findings that not the CAR T cells but other immune cells such as antigen-presenting cells might be responsible for the high IL-6 levels associated with CAR T-cell treatment. PMA/Ionomycin-stimulated T cells served as positive, untransduced T cells co-cultured with autologous B cells/blasts as negative control. To confirm the specific cytokine release of CAR T cells, an intracellular cytokine stain of CAR T cells after contact to a $CD19^+$ target cell line was performed for the two most relevant TH1 cytokines IFN-γ and TNF-α. A significant increase of IFN-γ- and TNF-α-positive cells was detected among CAR^+ cells after contact to the antigen, whereas CAR^- cells secreted IFN-γ and TNF-α to a significantly lower amount (Fig. 6c).

Discussion

Several phase I/II trials have demonstrated strong anti-leukemic potency of T cells genetically modified to express a chimeric antigen receptor (CAR) against the B-cell lineage antigen CD19 [5, 6]. Anti-CD19 CAR T cells are able to induce complete remissions in up to 95% of patients suffering from relapsed/refractory B-cell malignancies after several courses of second or even third/fourth line therapy [22]. A major hurdle that limited broad availability of this highly effective treatment was the lack of simplified production of a functionally active CAR T-cell product. In this study, we provide a solution to produce CAR T cells against $CD19^+$ malignancies in GMP centers with clean room grade B/C for closed steps and grade A for open steps.

We used challenging blood products from pediatric ALL patients with high variability in cellular composition

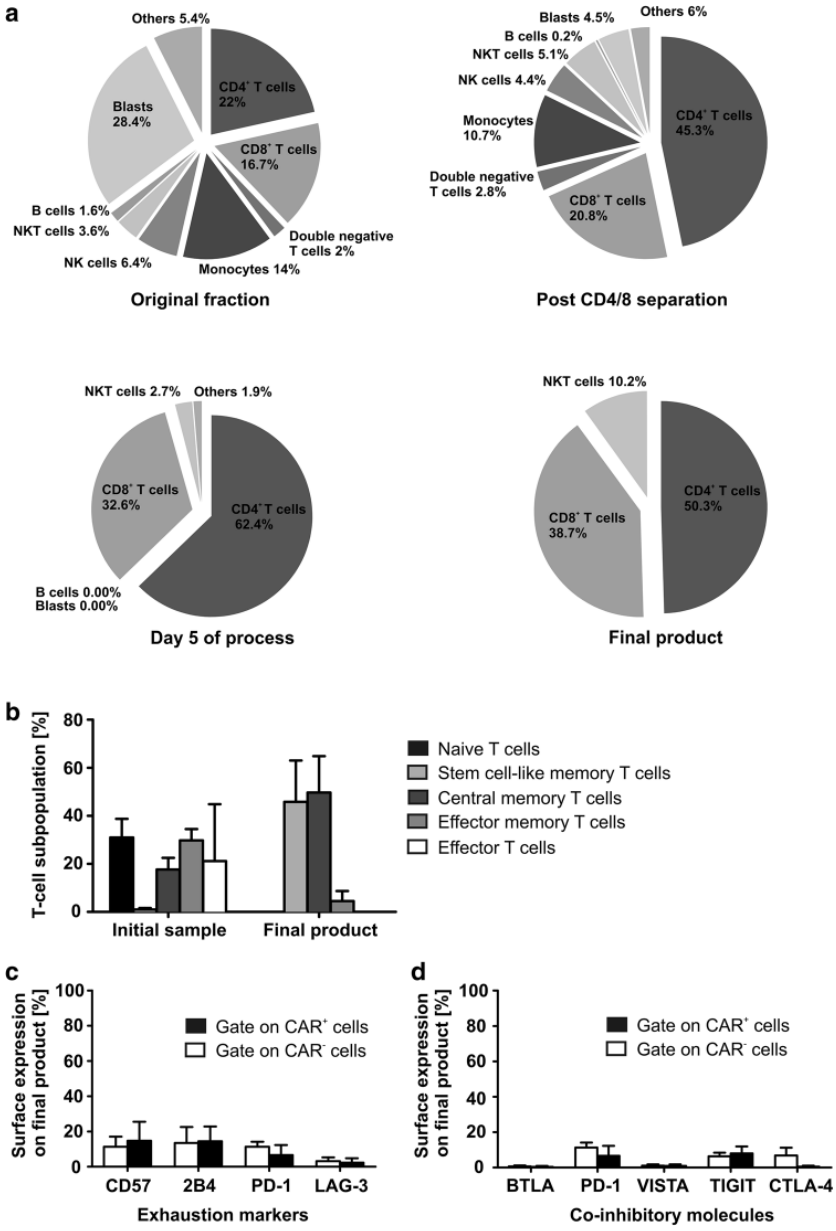


Fig. 4 Cellular composition and T-cell phenotype of CAR T cells. **a** Despite broad variety in cellular composition of the initial blood sample and after CD4/8 separation, a robust final product was achieved consisting of $CD4^+$, $CD8^+$ T cells and NKT Cells. No blasts or B cells were detectable on day 5 of the process and in the final product. **b** Despite a rather exhausted phenotype of the initial blood product, a Tcm and Tscm T-cell phenotype of the final product was reached. Effector T cells: $CD62L^-$, $CD45RO^-$, $CD95^+$; effector memory T cells: $CD62L^-$, $CD45RO^+$, $CD95^+$; central memory T cells: $CD62L^+$, $CD45RO^+$, $CD95^+$; stem cell-like memory T cells: $CD62L^+$, $CD45RO^-$, $CD95^+$; naïve T cells: $CD62L^+$, $CD45RO^-$, $CD95^-$. **c, d** Anti-CD19 CAR T cells were stained for extracellular expression of T-cell exhaustion/senescence markers and co-inhibitory molecules. No significant differences in expression between CAR^+ and CAR^- T cells were detectable. Wilcoxon signed rank test was performed to determine statistical significance

including high blast counts and low T-cell frequencies. A balanced and stable CD4/8 ratio of the final CAR T-cell product was achieved. No T-cell exhaustion was seen in the final T-cell product. The majority of T cells in the product showed a Tscm/Tcm phenotype determined by expression of CD62L and CD95 or additional expression of CD45RO, respectively. Although repeated stimulation does not reflect in vivo persistence and clinical outcome, we confirm a sustained proliferation after short term re-stimulation in vitro.

The generation of anti-CD19 CAR T cells is a time-consuming and technically challenging procedure consisting of T-cell separation, activation, transduction with a retro- or lentiviral vector and expansion. Current standard manufacturing protocols use large culture flasks or bioreactors for expansion and include many open steps with hands-on

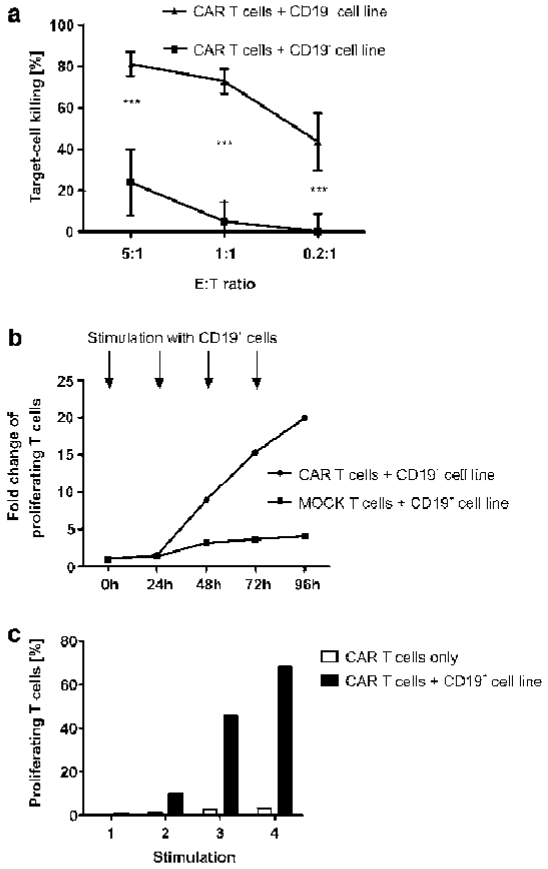


Fig. 5 Functionality analysis of CAR T cells. a CAR T cells showed dose-dependent killing of the CD19$^+$ leukemic target cell line Raji determined by flow cytometry-based functionality assay (n = 4 CAR T-cell products, technical duplicates or triplicates). CD19$^-$ cell lines U-266 and Molm-13 served as control (n = 3 CAR T-cell products, technical duplicates or triplicates). Two-tailed unpaired t test was performed to determine statistical significance. b Proliferation results of patient-derived CAR T cells: CAR T cells or untransduced T cells were co-cultured with CD19$^+$ cell line Jeko and re-stimulated with the same cell line every 24 h. Proliferation assay was performed before stimulation and every 24 h after stimulation. The figure shows fold change of proliferating CAR T cells after co-culture compared to T cells only. c Proliferation in percentage of proliferating cells 24 h after stimulation 1–4 compared to CAR T cells without stimulation

time of the personnel [19]. To facilitate CAR T-cell generation, the lentiviral transduction process on the CliniMACS Prodigy was previously described by Mock et al. and Lock et al. [20, 23]. Mock and colleagues used healthy donor leukapheresis to compare the performance of nanomatrix stimulation with conventional bead-based stimulation [20]. Lock et al. used an anti-CD20 CAR with a variety of starting materials (leukapheresis, buffy coat, a single sample whole blood—either derived from healthy donors, melanoma or adult diffuse large B-cell lymphoma patients) [23]. Here, we transfer the process to small blood samples of pediatric patients. In contrast to Mock et al. and Lock et al., we use PBMCs of pediatric patients suffering from acute lymphoblastic leukemia which are transduced with a second generation anti-CD19 CAR virus designed for clinical application. Encouraged by Lock and colleagues who already described one successful run with a blood sample derived from an adult melanoma patient [23], we used 100–120 ml of peripheral blood from pediatric ALL patients. 2/4 initial samples contained residual leukemic blasts. Since the transfer of the protocol towards a clinical ALL trial is planned, the present study was designed to achieve closest possible approximation of the starting material to reflect the clinical situation of the pediatric ALL patients. Due to ethical restrictions, for research use only, a leukapheresis from children has not been possible. For clinical studies, especially in patients who will not tolerate blood sampling of 100 ml or suffer from very low T-cell counts, a leukapheresis still might be inevitable to standardize T-cell counts and guarantee sufficient CAR T-cell numbers. Nevertheless, our work describes the potential of this process to generate functional CAR T-cell products even from very small, unstandardized and challenging pediatric ALL patient samples.

T-cell separation, activation, transduction, expansion and harvest were performed in a closed sterile tubing set. Open steps are isolation of PBMCs, preparation of buffers, vector and media and cryopreservation of the final product. All reagents are connected to the device by spiking or sterile tube welding. Labeling of the cells with anti-CD4/-CD8 microbeads was performed in the culture chamber followed by separation with the incorporated MACS column and the magnet of the device. Small scale experiments from healthy donors showed no significant impact of CD4/CD8 separation concerning expansion, transduction and T-cell phenotype. Nevertheless, B-cell counts of the starting fraction were significantly higher in the un-separated samples (Supplementary Figure 3). Especially for patients with residual leukemic blasts, CD4/CD8 separation is regarded as a safety procedure to decrease blast counts in the culture. Although sufficient enrichment of the patient-derived CD4/CD8 T cells was observed after magnetic separation, effort needs to be spent on improving CD4/8 purity especially of samples with high blast frequencies. Nevertheless, already five days after separation, no malignant cells or B cells were detectable in the culture chamber anymore, proofing complete elimination of leukemic cells and thus excluding a re-infusion of leukemic cells. Activation was performed using a modified CD3/CD28 T-cell reagent, which is not based on conventional magnetic bead technology but uses a polymeric nanomatrix structure which is conjugated to CD3/28 agonist. Thus, it does not require a magnetic bead removal step but is washed out by centrifugation two days

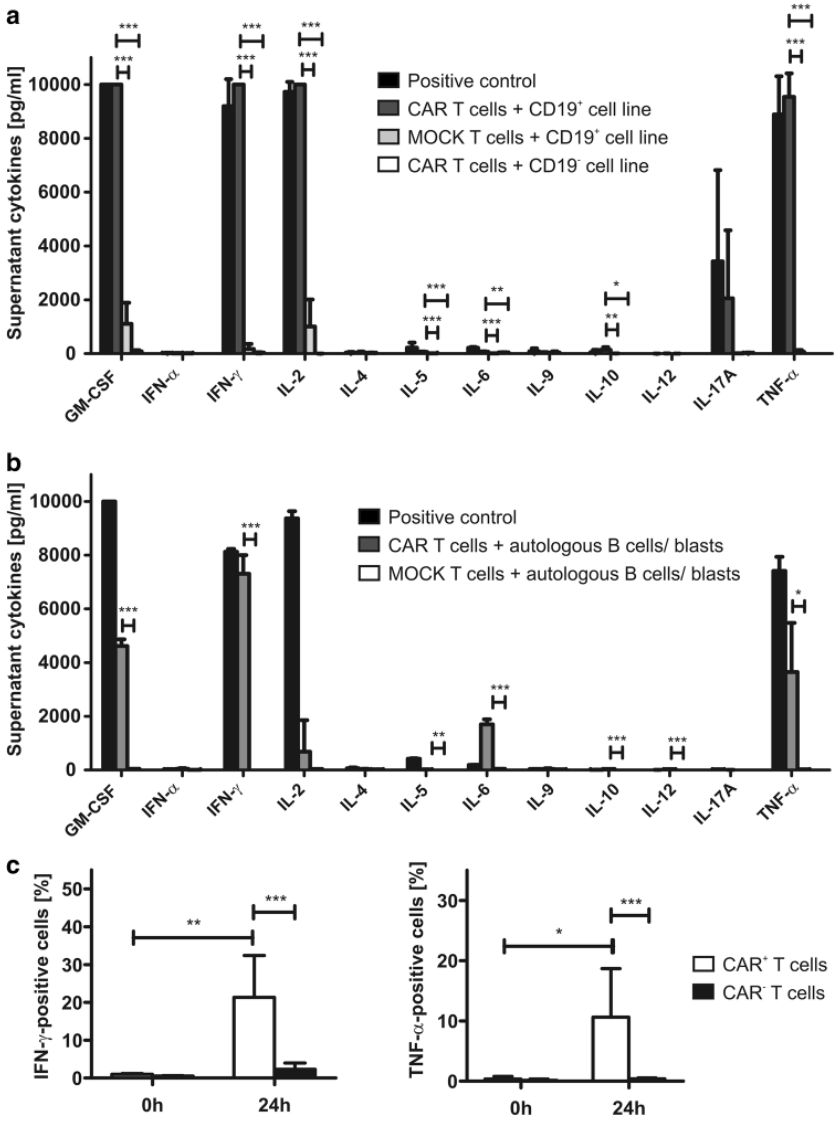


Fig. 6 Functionality and target cell-dependent TH1 cytokine release of CAR T cells. **a** CAR T cells were co-cultured with the $CD19^+$ target cell line Raji. After 24 h, supernatant was analyzed for cytokine concentrations using a flow cytometry-based assay. Untransduced T cells of the same donor and $CD19^-$ cell lines (U-266 and Molm-13) served as negative, stimulation with PMA/ionomycine as positive control ($n = 3$ CAR T-cell products, technical duplicates or triplicates). **b** Exemplary cytokine release assay of run CAR003: CAR T cells were co-cultured with the negative fraction of CD4/CD8 separation performed on day 0 of the process. After 24 h, supernatant was analyzed for cytokine concentrations using a flow cytometry-based assay. Untransduced T cells of the same patient co-cultured with the same autologous target-cell fraction served as negative, stimulation with PMA/ionomycine as positive control (technical triplicates). **c** Intracellular cytokine stain of anti-CD19 CAR T cells for IFN-γ and TNF-α was performed 24 h after contact to the $CD19^+$ cell line Raji. A significant increase of IFN-γ and TNF-α positivity was detected for CAR^+ cells ($n = 4$ CAR T-cell products). Ratio paired two-tailed *t* test was performed to determine statistical significance

later. This procedure is anyway required to remove residual lentivirus from the culture one day after transduction. As lentiviral vectors are known to have a lower risk for mutational oncogenesis than γ-retroviral vectors [24], transduction was performed with a lentiviral vector encoding the anti-CD19 CAR one day after activation and yielded sufficient transduction rates. Because the process was initially standardized for 1×10^8 T cells on day 0, a viral concentration of 2×10^8 particles/100 ml was used to reach an MOI of 2. Here, due to extremely high variety of T-cell counts and to achieve a constant concentration of viral particles, we transduced with a standard dose of 2×10^8 viral particles, irrespective of T-cell counts. Estimated MOI rates and corresponding transduction rates are shown in Supplementary Table 2. Despite high variability in MOI rates, the vector copy number did not exceed 2.3 per T cell, and thus met the authority's requirements. For use in clinical trials the T-cell count can be adjusted to 1×10^8 T cells/100 ml with a viral concentration of 2×10^8 particles/100 ml, resulting in a standardized MOI of 2. In contrast to other manufacturing protocols [25], CD4 and CD8 T cells were not cultured separately but within one culture chamber in the presence of IL-7 and IL-15. T-cell proliferation within the culture was up to > 100 fold despite challenging blood samples with low

initial T-cell counts. Nevertheless, especially in the presence of blasts, T-cell numbers might decrease to levels below detection limit and could only be identified in the sampling pouch after additional re-concentration. This decrease in T-cell count might be caused by blast destruction and the formation of free DNA. Fortunately, T-cell counts robustly recovered from day 5 of the process until final formulation.

The final CAR T-cell product described here consists of highly functional CAR T cells which were able to specifically lyse $CD19^+$ cell lines and secrete IFN-γ and TNF-α upon target-cell contact. Interestingly, IL-6 was not released by CAR T cells co-cultured with leukemic target-cell lines underlining recent findings that not CAR T cells but antigen-presenting cells might be responsible for high IL-6 levels observed during CAR-triggered cytokine release syndrome [26]. We recently reported that the expression of exhaustion/activation marker PD-1 is significantly higher on ALL patients' T cells compared to healthy donors and is induced by T-cell attack against blasts [17]. The CAR T cells produced with the current protocol expressed rather low levels of inhibitory checkpoint molecules such as PD-1, CTLA-4, 2B4 and LAG-3 on their surface indicating a low susceptibility for inhibitory signals derived by the malignant cell and confirming their non-exhausted, highly functional phenotype.

In conclusion, a partly automated protocol for GMP-compatible generation of CAR T cells in a closed system was developed which yields high CAR T-cell counts even from small pediatric patient blood samples. In case a leukapheresis cannot be performed due to ethical restrictions or the current clinical condition of the patient, this protocol enables the generation of a sufficient number of CAR T cells even from 100 to 120 ml peripheral blood. The final CAR T-cell product has a robust CD4/8 composition with a favorable Tcm and Tscm phenotype, good functionality and low expression of inhibitory checkpoint molecules. The manufacturing protocol described here will enable safe multi-center manufacturing of CAR T cells. The process fulfils current requirements of regulatory authorities and simplifies the complex procedure of CAR manufacturing while minimizing the risk for contamination and operator-to-operator variability. In vivo efficacy and safety of the cells produced here will be evaluated in subsequent clinical trials.

Acknowledgements The authors thank all patients and their parents for participating in the study. Nadine Stoll, Tanja Weisser, Nicola Habjan, Florian Jurgeleit, Carola Barth and Daniela Mauer are acknowledged for excellent technical assistance. The authors thank Katharina Drechsel and Nadine Mockel-Tenbrinck for helpful advice.

Author contributions Experiments were designed by TF, FB and ADK; the automated process was developed by ADK and MA; patient samples were provided by TF, SW and MD; experiments were performed by FB, DS and TK; JF set up experiments and provided protocols; RL provided healthy donor starting fractions and human serum. Data analysis was done by FB, DS and TF; the manuscript was written by FB and TF and was reviewed by all co-authors.

Funding This work was supported by Elterninitiative Ebersberg, Elterninitative Intern3 and Bettina Braeu Stiftung, Adler Stiftung and the Care for Rare Foundation. Miltenyi Biotec provided reagents free of charge.

Compliance with ethical standards

Conflict of interest Andrew Didier Kaiser and Mario Assenmacher are employees of Miltenyi Biotec. This work has been performed as a collaboration between Tobias Feuchtinger, Franziska Blaeschke and Miltenyi Biotec. Miltenyi Biotec provided reagent free of charge. All other authors declare that they have no conflict of interest.

Ethical approval and ethical standards This study was approved by the Institutional Ethical Review Board ("Ethikkommission bei der LMU München"), approval number 435 – 15, and was performed in accordance with the Declaration of Helsinki.

Informed consent Patients/their representatives gave written informed consent according to the guidelines and approval of the Institutional Ethical Review Board.

Cell line authentication Cell lines Raji, Jeko, Molm-13, U-266 were routinely tested for identity by short-tandem repeat analyses (DSMZ, Braunschweig, Germany).

References

1. Blaeschke F, Kaeuferle T, Feucht J, Weber D, Lotfi R, Kaiser A, Assenmacher M, Doering M, Feuchtinger T (2016) Defined central memory and stem memory T cell phenotype of CD4 and CD8 CAR T cells for the treatment of $CD19^+$ acute lymphoblastic leukemia in an automated closed system. In: 58th ASH annual meeting and exposition. Blood, 128(22) **(Abstract 4558)**
2. Stenger D, Blaeschke F, Kaeuferle T, Willier S, Lotfi R, Kaiser A, Assenmacher M, Doering M, Feucht J, Feuchtinger T (2017) Automated generation of central memory and stem cell-like memory CD19-specific CAR T cells in a closed, GMP compatible system. In: Cellular therapy, international symposium Erlangen, Erlangen **(Abstract 134)**
3. Blaeschke F, Stenger D, Kaeuferle T, Willier S, Lotfi R, Kaiser A, Assenmacher M, Doering M, Feucht J, Feuchtinger T (2017) CD19-specific CAR T cells with a central memory and stem memory phenotype—automated generation in a closed, GMP-compatible system from peripheral blood of pediatric patients with acute lymphoblastic leukemia. In: 43rd annual meeting of the European Society for Blood and Marrow Transplantation (EBMT), Marseille **(Abstract)**
4. Blaeschke F, Stenger D, Kaeuferle T, Willier S, Lotfi R, Kaiser A, Assenmacher M, Doering M, Feucht J, Feuchtinger T (2017) Induction of a central memory and stem cell memory phenotype in functionally active $CD4^+$ and $CD8^+$ CAR T cells produced in an automated GMP system for the treatment of $CD19^+$ acute lymphoblastic leukemia. In: Annual meeting of Paediatrische Arbeitsgemeinschaft fuer Stammzelltransplantation and Zelltherapie (PAS&ZT), Hamburg **(Abstract)**
5. Lee DW, Kochenderfer JN, Stetler-Stevenson M, Cui YK, Delbrook C, Feldman SA, Fry TJ, Orentas R, Sabatino M, Shah NN, Steinberg SM, Stroncek D, Tschernia N, Yuan C, Zhang H, Zhang

L, Rosenberg SA, Wayne AS, Mackall CL (2015) T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. Lancet 385(9967):517–528. https://doi.org/10.1016/S0140-6736(14)61403-3

6. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, Chew A, Gonzalez VE, Zheng Z, Lacey SF, Mahnke YD, Melenhorst JJ, Rheingold SR, Shen A, Teachey DT, Levine BL, June CH, Porter DL, Grupp SA (2014) Chimeric antigen receptor T cells for sustained remissions in leukemia. N Engl J Med 371(16):1507–1517. https://doi.org/10.1056/NEJMoa1407222
7. Kochenderfer JN, Dudley ME, Kassim SH, Somerville RP, Carpenter RO, Stetler-Stevenson M, Yang JC, Phan GQ, Hughes MS, Sherry RM, Raffeld M, Feldman S, Lu L, Li YF, Ngo LT, Goy A, Feldman T, Spaner DE, Wang ML, Chen CC, Kranick SM, Nath A, Nathan DA, Morton KE, Toomey MA, Rosenberg SA (2015) Chemotherapy-refractory diffuse large B-cell lymphoma and indolent B-cell malignancies can be effectively treated with autologous T cells expressing an anti-CD19 chimeric antigen receptor. J Clin Oncol 33(6):540–549. https://doi.org/10.1200/JCO.2014.56.2025
8. Porter DL, Levine BL, Kalos M, Bagg A, June CH (2011) Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med 365(8):725–733. https://doi.org/10.1056/NEJMoa1103849
9. Orlowski RJ, Porter DL, Frey NV (2017) The promise of chimeric antigen receptor T cells (CARTs) in leukaemia. Br J Haematol 177(1):13–26. https://doi.org/10.1111/bjh.14475
10. Maude S, Barrett DM (2016) Current status of chimeric antigen receptor therapy for haematological malignancies. Br J Haematol 172(1):11–22. https://doi.org/10.1111/bjh.13792
11. Sadelain M, Brentjens R, Riviere I (2013) The basic principles of chimeric antigen receptor design. Cancer Discov 3(4):388–398. https://doi.org/10.1158/2159-8290.CD-12-0548
12. Sotillo E, Barrett DM, Black KL, Bagashev A, Oldridge D, Wu G, Sussman R, Lanauze C, Ruella M, Gazzara MR, Martinez NM, Harrington CT, Chung EY, Perazzelli J, Hofmann TJ, Maude SL, Raman P, Barrera A, Gill S, Lacey SF, Melenhorst JJ, Allman D, Jacoby E, Fry T, Mackall C, Barash Y, Lynch KW, Maris JM, Grupp SA, Thomas-Tikhonenko A (2015) Convergence of acquired mutations and alternative splicing of CD19 enables resistance to CART-19 immunotherapy. Cancer Discov 5(12):1282–1295. https://doi.org/10.1158/2159-8290.CD-15-1020
13. Jacoby E, Nguyen SM, Fountaine TJ, Welp K, Gryder B, Qin H, Yang Y, Chien CD, Seif AE, Lei H, Song YK, Khan J, Lee DW, Mackall CL, Gardner RA, Jensen MC, Shern JF, Fry TJ (2016) CD19 CAR immune pressure induces B-precursor acute lymphoblastic leukaemia lineage switch exposing inherent leukaemic plasticity. Nat Commun 7:12320. https://doi.org/10.1038/ncomms12320
14. Wang X, Popplewell LL, Wagner JR, Naranjo A, Blanchard MS, Mott MR, Norris AP, Wong CW, Urak RZ, Chang WC, Khaled SK, Siddiqi T, Budde LE, Xu J, Chang B, Gidwaney N, Thomas SH, Cooper LJ, Riddell SR, Brown CE, Jensen MC, Forman SJ (2016) Phase 1 studies of central memory-derived CD19 CAR T-cell therapy following autologous HSCT in patients with B-cell NHL. Blood 127(24):2980–2990. https://doi.org/10.1182/blood-2015-12-686725
15. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, Almeida JR, Gostick E, Yu Z, Carpenito C, Wang E, Douek DC, Price DA, June CH, Marincola FM, Roederer M, Restifo NP (2011) A human memory T cell subset with stem cell-like properties. Nat Med 17(10):1290–1297. https://doi.org/10.1038/nm.2446
16. Biasco L, Scala S, Basso Ricci L, Dionisio F, Baricordi C, Calabria A, Giannelli S, Cieri N, Barzaghi F, Pajno R, Al-Mousa H, Scarselli A, Cancrini C, Bordignon C, Roncarolo MG, Montini E, Bonini C, Aiuti A (2015) In vivo tracking of T cells in humans unveils decade-long survival and activity of genetically modified T memory stem cells. Sci Transl Med 7(273):273ra213. https://doi.org/10.1126/scitranslmed.3010314
17. Feucht J, Kayser S, Gorodezki D, Hamieh M, Doring M, Blaeschke F, Schlegel P, Bosmuller H, Quintanilla-Fend L, Ebinger M, Lang P, Handgretinger R, Feuchtinger T (2016) T-cell responses against $CD19^+$ pediatric acute lymphoblastic leukemia mediated by bispecific T-cell engager (BiTE) are regulated contrarily by PD-L1 and CD80/CD86 on leukemic blasts. Oncotarget 7(47):76902–76919. https://doi.org/10.18632/oncotarget.12357
18. Cherkassky L, Morello A, Villena-Vargas J, Feng Y, Dimitrov DS, Jones DR, Sadelain M, Adusumilli PS (2016) Human CAR T cells with cell-intrinsic PD-1 checkpoint blockade resist tumor-mediated inhibition. J Clin Investig 126(8):3130–3144. https://doi.org/10.1172/JCI83092
19. Hollyman D, Stefanski J, Przybylowski M, Bartido S, Borquez-Ojeda O, Taylor C, Yeh R, Capacio V, Olszewska M, Hosey J, Sadelain M, Brentjens RJ, Riviere I (2009) Manufacturing validation of biologically functional T cells targeted to CD19 antigen for autologous adoptive cell therapy. J Immunother 32(2):169–180. https://doi.org/10.1097/CJI.0b013e318194a6e8
20. Mock U, Nickolay L, Philip B, Cheung GW, Zhan H, Johnston IC, Kaiser AD, Peggs K, Pule M, Thrasher AJ, Qasim W (2016) Automated manufacturing of chimeric antigen receptor T cells for adoptive immunotherapy using CliniMACS prodigy. Cytotherapy 18(8):1002–1011. https://doi.org/10.1016/j.jcyt.2016.05.009
21. Schneider D, Xiong Y, Wu D, Nlle V, Schmitz S, Haso W, Kaiser A, Dropulic B, Orentas RJ (2017) A tandem CD19/CD20 CAR lentiviral vector drives on-target and off-target antigen modulation in leukemia cell lines. J Immunother Cancer 5:42. https://doi.org/10.1186/s40425-017-0246-1
22. Gardner RA, Finney O, Annesley C, Brakke H, Summers C, Leger K, Bleakley M, Brown C, Mgebroff S, Kelly-Spratt KS, Hoglund V, Lindgren C, Oron AP, Li D, Riddell SR, Park JR, Jensen MC (2017) Intent-to-treat leukemia remission by CD19 CAR T cells of defined formulation and dose in children and young adults. Blood 129(25):3322–3331. https://doi.org/10.1182/blood-2017-02-769208
23. Lock D, Mockel-Tenbrinck N, Drechsel K, Barth C, Mauer D, Schaser T, Kolbe C, Al Rawashdeh W, Brauner J, Hardt O, Pflug N, Holtick U, Borchmann P, Assenmacher M, Kaiser A (2017) Automated manufacturing of potent CD20-directed chimeric antigen receptor T cells for clinical use. Hum Gene Ther 28(10):914–925. https://doi.org/10.1089/hum.2017.111
24. Montini E, Cesana D, Schmidt M, Sanvito F, Bartholomae CC, Ranzani M, Benedicenti F, Sergi LS, Ambrosi A, Ponzoni M, Doglioni C, Di Serio C, von Kalle C, Naldini L (2009) The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. J Clin Investig 119(4):964–975. https://doi.org/10.1172/JCI37630
25. Turtle CJ, Hanafi LA, Berger C, Gooley TA, Cherian S, Hudecek M, Sommermeyer D, Melville K, Pender B, Budiarto TM, Robinson E, Steevens NN, Chaney C, Soma L, Chen X, Yeung C, Wood B, Li D, Cao J, Heimfeld S, Jensen MC, Riddell SR, Maloney DG (2016) CD19 CAR-T cells of defined $CD4^+$:$CD8^+$ composition in adult B cell ALL patients. J Clin Investig 126(6):2123–2138. https://doi.org/10.1172/JCI85309
26. Barrett DM, Singh N, Hofmann TJ, Gershenson Z, Grupp SA (2016) Interleukin 6 is not made by chimeric antigen receptor T cells and does not impact their function. In: 58th ASH annual meeting and exposition. Blood, 128(22) **(Abstract 654)**

Affiliations

Franziska Blaeschke[1] · Dana Stenger[1] · Theresa Kaeuferle[1] · Semjon Willier[1] · Ramin Lotfi[2,3] · Andrew Didier Kaiser[4] · Mario Assenmacher[4] · Michaela Döring[1,5] · Judith Feucht[5,6] · Tobias Feuchtinger[1]

✉ Tobias Feuchtinger
tobias.feuchtinger@med.uni-muenchen.de

[1] Department of Pediatric Hematology, Oncology, Hemostaseology and Stem Cell Transplantation, Dr. von Hauner University Children's Hospital, Ludwig Maximilian University Munich, Lindwurmstrasse 4, 80337 Munich, Germany

[2] Institute for Transfusion Medicine, University Hospital Ulm, Ulm, Germany

[3] Institute for Clinical Transfusion Medicine and Immunogenetics Ulm, German Red Cross Blood Services Baden-Württemberg-Hessen, Ulm, Germany

[4] Miltenyi Biotec, Bergisch Gladbach, Germany

[5] Department I - General Pediatrics, Hematology/Oncology, University Hospital Tübingen, Children's Hospital, Tübingen, Germany

[6] Memorial Sloan Kettering Cancer Center, Center for Cell Engineering, New York, USA

4. Paper II

Material from: Blaeschke et al., "Leukemia-induced dysfunctional TIM-3$^+$CD4$^+$ bone marrow T cells increase risk of relapse in pediatric B-precursor ALL patients", Leukemia, published 2020, Springer Nature

Leukemia
https://doi.org/10.1038/s41375-020-0793-1

ARTICLE

Acute lymphoblastic leukemia

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

Franziska Blaeschke[1] · Semjon Willier[1] · Dana Stenger[1] · Mareike Lepenies[1] · Martin A. Horstmann[2] · Gabriele Escherich[2] · Martin Zimmermann[3] · Francisca Rojas Ringeling[4] · Stefan Canzar[4] · Theresa Kaeuferle[1] · Meino Rohlfs[1] · Vera Binder[1] · Christoph Klein[1,4] · Tobias Feuchtinger[1]

Received: 24 September 2019 / Revised: 29 February 2020 / Accepted: 3 March 2020

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.

✉ Tobias Feuchtinger
tobias.feuchtinger@med.uni-muenchen.de

[1] Dr. von Hauner Children's Hospital, University Hospital, Ludwig Maximilian University, 80337 Munich, Germany

[2] Clinic of Pediatric Hematology and Oncology, University Medical Center Hamburg-Eppendorf, 20251 Hamburg, Germany

[3] Department of Pediatric Hematology and Oncology, Hannover Medical School, 30625 Hannover, Germany

[4] Gene Center, Ludwig Maximilian University Munich, 81377 Munich, Germany

Published online: 13 March 2020

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 × *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).

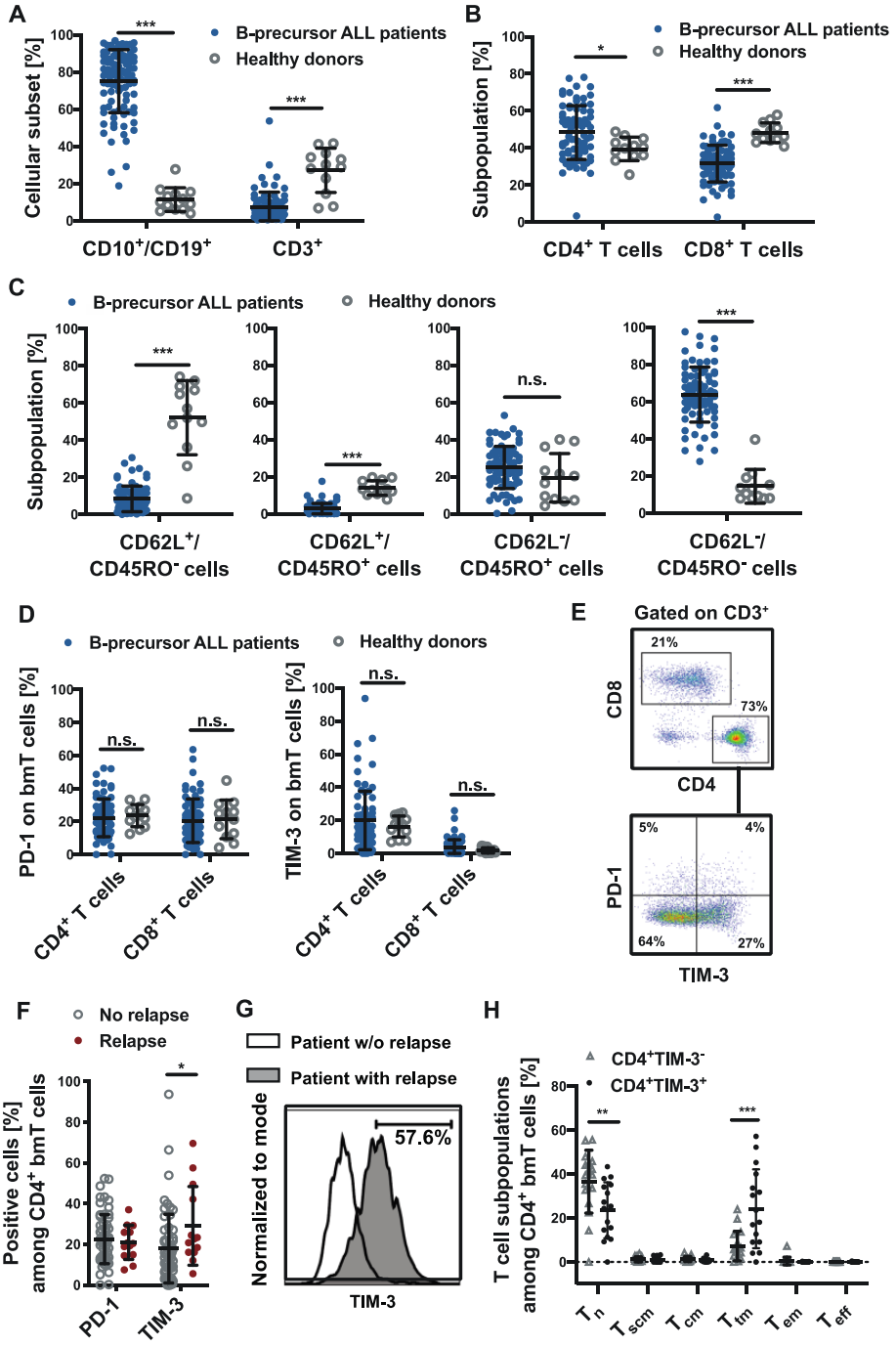
Statistics

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,

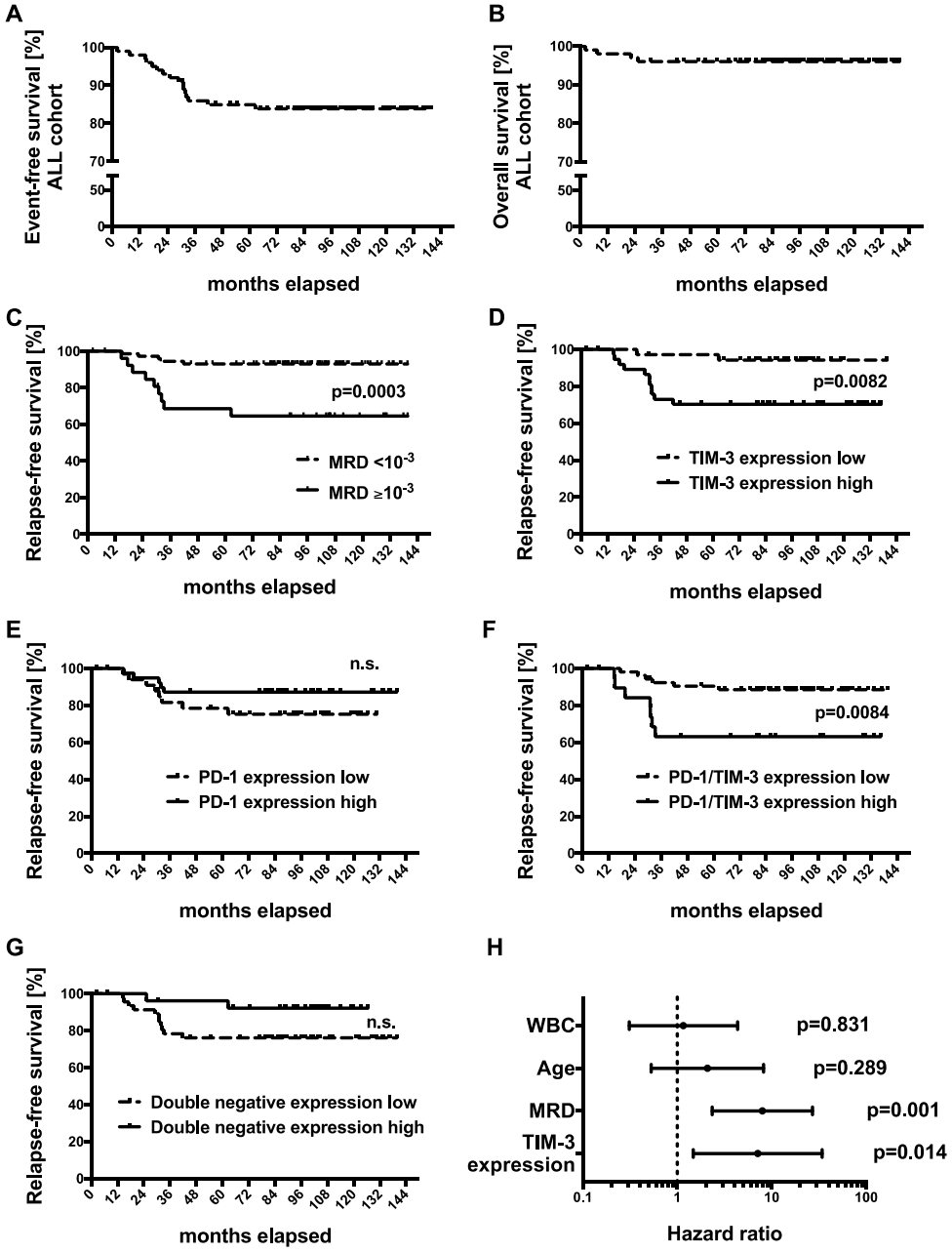


Fig. 2 Expression of exhaustion marker TIM-3 on CD4^{+} bone marrow T cells is a risk factor for relapse of pediatric B-precursor ALL. The ALL cohort showed an **a** event-free and **b** overall survival rate of 84 and 96% respectively. **c** High MRD levels after induction chemotherapy correlates with 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

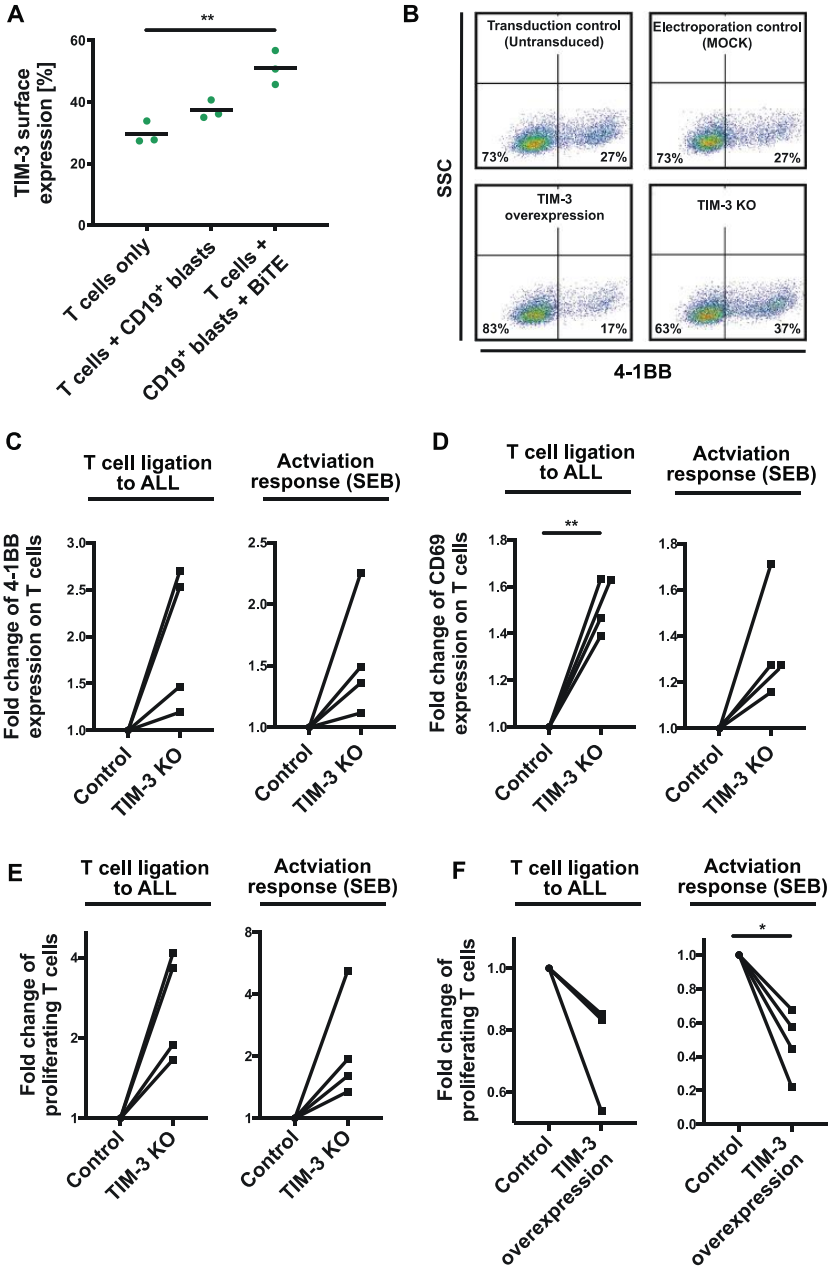
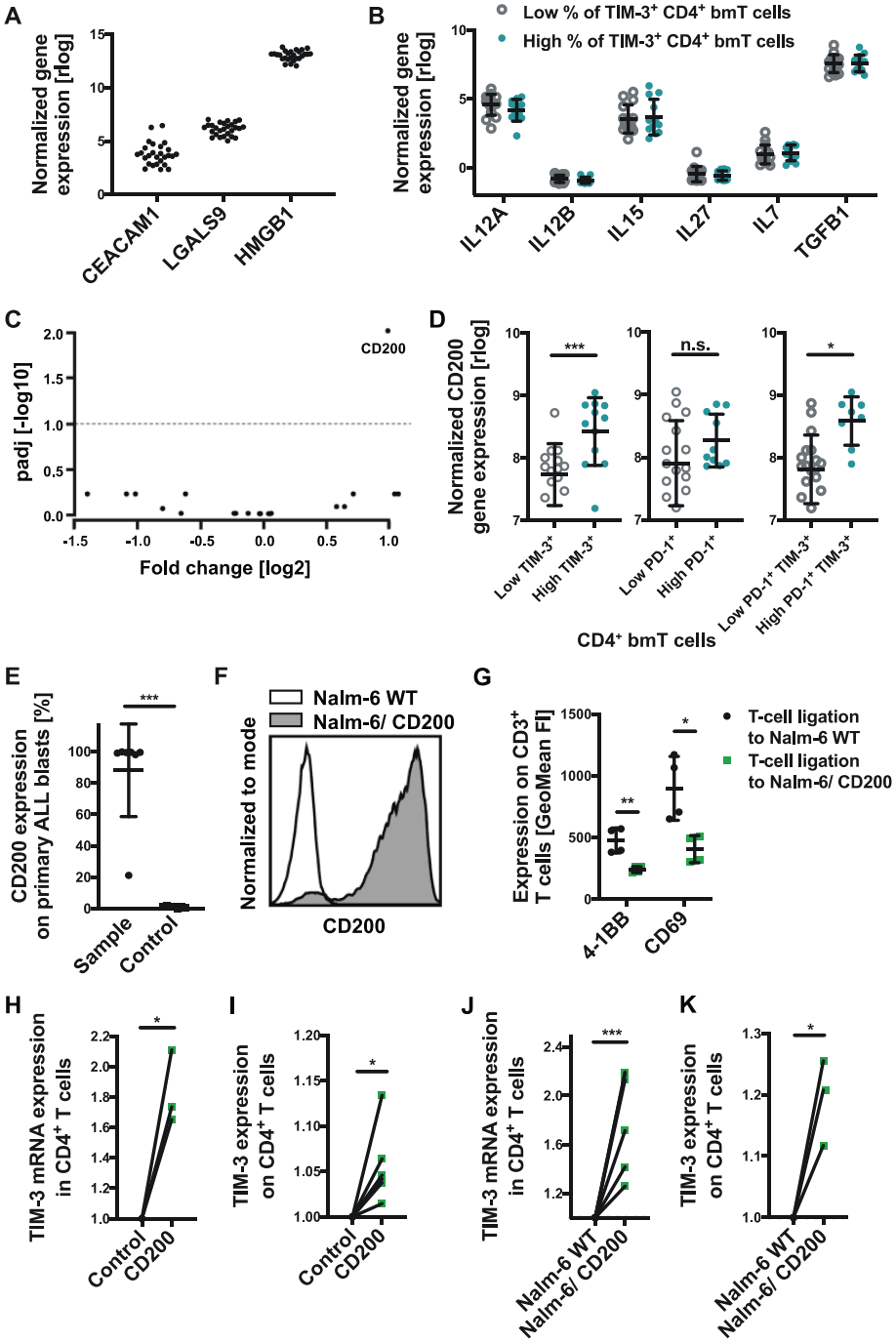


Fig. 3 Leukemia is associated with upregulation of TIM-3 on T cells leading to reduced activation and proliferation potential of T cells. a T cells were left untreated or co-cultured with CD19^{+} target cells with/without blinatumomab that causes a direct T cell to blast contact. TIM-3 expression is 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 immunemodulatory 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.

Acknowledgements The authors thank the patients and their families for participating in the study. We thank Tanja Weißer, Nicola Habjan and Nadine Stoll for excellent technical assistance. This work was supported by the Elterninitiative Ebersberg, Elterninitiative Intern 3, Bettina Braeu Stiftung, Gesellschaft für KinderKrebsForschung e.V. and Dr. Sepp und Hanne Sturm Gedaechtnisstiftung. S.W. was supported by the Else-Kröner-Fresenius Stiftung and D.S. was supported by the German Cancer Research Center/German Cancer Consortium (DKTK).

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.

References

1. Moricke A, Zimmermann M, Reiter A, Henze G, Schrauder A, Gadner H, et al. Long-term results of five consecutive trials in childhood acute lymphoblastic leukemia performed by the ALL-BFM study group from 1981 to 2000. Leukemia. 2010;24:265–84.
2. Ladanyi A, Somlai B, Gilde K, Fejos Z, Gaudi I, Timar J. T-cell activation marker expression on tumor-infiltrating lymphocytes as prognostic factor in cutaneous malignant melanoma. Clin Cancer Res. 2004;10:521–30.
3. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer. 2012;12:252–64.
4. Kleffel S, Posch C, Barthel SR, Mueller H, Schlapbach C, Guenova E, et al. Melanoma cell-intrinsic PD-1 receptor functions promote tumor growth. Cell. 2015;162:1242–56.
5. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, et al. Combined Nivolumab and Ipilimumab or monotherapy in untreated melanoma. N Engl J Med. 2015;373:23–34.
6. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, et al. Signatures of mutational processes in human cancer. Nature. 2013;500:415–21.
7. Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. N Engl J Med. 2014;371:2189–99.
8. Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T, et al. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. Nature. 2002;415:536–41.
9. Mujib S, Jones RB, Lo C, Aidarus N, Clayton K, Sakhdari A, et al. Antigen-independent induction of Tim-3 expression on human T cells by the common gamma-chain cytokines IL-2, IL-7, IL-15, and IL-21 is associated with proliferation and is dependent on the phosphoinositide 3-kinase pathway. J Immunol. 2012;188:3745–56.
10. Yang ZZ, Grote DM, Ziesmer SC, Niki T, Hirashima M, Novak AJ, et al. IL-12 upregulates TIM-3 expression and induces T cell exhaustion in patients with follicular B cell non-Hodgkin lymphoma. J Clin Invest. 2012;122:1271–82.
11. Zhu C, Sakuishi K, Xiao S, Sun Z, Zaghouani S, Gu G, et al. An IL-27/NFIL3 signalling axis drives Tim-3 and IL-10 expression and T-cell dysfunction. Nat Commun. 2015;6:6072.
12. Wiener Z, Kohalmi B, Pocza P, Jeager J, Tolgyesi G, Toth S, et al. TIM-3 is expressed in melanoma cells and is upregulated in TGF-beta stimulated mast cells. J Invest Dermatol. 2007;127:906–14.
13. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. Nat Immunol. 2005;6:1245–52.
14. Chiba S, Baghdadi M, Akiba H, Yoshiyama H, Kinoshita I, Dosaka-Akita H, et al. Tumor-infiltrating DCs suppress nucleic acid-mediated innate immune responses through interactions between the receptor TIM-3 and the alarmin HMGB1. Nat Immunol. 2012;13:832–42.
15. Huang YH, Zhu C, Kondo Y, Anderson AC, Gandhi A, Russell A, et al. CEACAM1 regulates TIM-3-mediated tolerance and exhaustion. Nature. 2015;517:386–90.
16. Sabatos CA, Chakravarti S, Cha E, Schubart A, Sanchez-Fueyo A, Zheng XX, et al. Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. Nat Immunol. 2003;4:1102–10.
17. Sanchez-Fueyo A, Tian J, Picarella D, Domenig C, Zheng XX, Sabatos CA, et al. Tim-3 inhibits T helper type 1-mediated auto- and alloimmune responses and promotes immunological tolerance. Nat Immunol. 2003;4:1093–101.
18. Jin HT, Anderson AC, Tan WG, West EE, Ha SJ, Araki K, et al. Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. Proc Natl Acad Sci USA. 2010;107:14733–8.
19. Jones RB, Ndhlovu LC, Barbour JD, Sheth PM, Jha AR, Long BR, et al. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. J Exp Med. 2008;205:2763–79.
20. Fourcade J, Sun Z, Benallaoua M, Guillaume P, Luescher IF, Sander C, et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. J Exp Med. 2010;207:2175–86.
21. Gao X, Zhu Y, Li G, Huang H, Zhang G, Wang F, et al. TIM-3 expression characterizes regulatory T cells in tumor tissues and is associated with lung cancer progression. PLoS ONE. 2012;7:e30676.
22. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: co-inhibitory receptors with specialized functions in immune regulation. Immunity. 2016;44:989–1004.
23. Noviello M, Manfredi F, Ruggiero E, Perini T, Oliveira G, Cortesi F, et al. Bone marrow central memory and memory stem T-cell exhaustion in AML patients relapsing after HSCT. Nat Commun. 2019;10:1065.
24. Goncalves Silva I, Yasinska IM, Sakhnevych SS, Fiedler W, Wellbrock J, Bardelli M, et al. The Tim-3-galectin-9 secretory pathway is involved in the immune escape of human acute myeloid leukemia cells. EBioMedicine. 2017;22:44–57.
25. Hohtari H, Bruck O, Blom S, Turkki R, Sinisalo M, Kovanen PE, et al. Immune cell constitution in bone marrow microenvironment predicts outcome in adult ALL. Leukemia. 2019;33:1570–82.
26. Chicaybam L, Barcelos C, Peixoto B, Carneiro M, Limia CG, Redondo P, et al. An efficient electroporation protocol for the genetic modification of mammalian cells. Front Bioeng Biotechnol. 2016;4:99.
27. Feucht J, Kayser S, Gorodezki D, Hamieh M, Doring M, Blaeschke F, et al. T-cell responses against CD19+ pediatric acute lymphoblastic leukemia mediated by bispecific T-cell engager (BiTE) are regulated contrarily by PD-L1 and CD80/CD86 on leukemic blasts. Oncotarget. 2016;7:76902–19.
28. Qin H, Ishii K, Nguyen S, Su PP, Burk CR, Kim BH, et al. Murine pre-B-cell ALL induces T-cell dysfunction not fully reversed by introduction of a chimeric antigen receptor. Blood. 2018;132:1899–910.
29. Liu L, Chang YJ, Xu LP, Zhang XH, Wang Y, Liu KY, et al. T cell exhaustion characterized by compromised MHC class I and II restricted cytotoxic activity associates with acute B lymphoblastic leukemia relapse after allogeneic hematopoietic stem cell transplantation. Clin Immunol. 2018;190:32–40.
30. Tang R, Rangachari M, Kuchroo VK. Tim-3: A co-receptor with diverse roles in T cell exhaustion and tolerance. Semin Immunol. 2019;42:101302.
31. Pui CH, Yang JJ, Hunger SP, Pieters R, Schrappe M, Biondi A, et al. Childhood acute lymphoblastic leukemia: progress through collaboration. J Clin Oncol. 2015;33:2938–48.
32. Wright GJ, Jones M, Puklavec MJ, Brown MH, Barclay AN. The unusual distribution of the neuronal/lymphoid cell surface CD200 (OX2) glycoprotein is conserved in humans. Immunology. 2001;102:173–9.
33. Ring EK, Markert JM, Gillespie GY, Friedman GK. Checkpoint proteins in pediatric brain and extracranial solid tumors: opportunities for immunotherapy. Clin Cancer Res. 2017;23:342–50.
34. McWhirter JR, Kretz-Rommel A, Saven A, Maruyama T, Potter KN, Mockridge CI, et al. Antibodies selected from combinatorial libraries block a tumor antigen that plays a key

role in immunomodulation. Proc Natl Acad Sci USA. 2006;103:1041–6.
35. Stumpfova M, Ratner D, Desciak EB, Eliezri YD, Owens DM. The immunosuppressive surface ligand CD200 augments the metastatic capacity of squamous cell carcinoma. Cancer Res. 2010;70:2962–72.
36. Petermann KB, Rozenberg GI, Zedek D, Groben P, McKinnon K, Buehler C, et al. CD200 is induced by ERK and is a potential therapeutic target in melanoma. J Clin Invest. 2007;117:3922–9.
37. Palumbo GA, Parrinello N, Fargione G, Cardillo K, Chiarenza A, Berretta S, et al. CD200 expression may help in differential diagnosis between mantle cell lymphoma and B-cell chronic lymphocytic leukemia. Leuk Res. 2009;33:1212–6.
38. Coles SJ, Gilmour MN, Reid R, Knapper S, Burnett AK, Man S, et al. The immunosuppressive ligands PD-L1 and CD200 are linked in AML T-cell immunosuppression: identification of a new immunotherapeutic synapse. Leukemia. 2015;29:1952–4.
39. Coles SJ, Hills RK, Wang EC, Burnett AK, Man S, Darley RL, et al. Expression of CD200 on AML blasts directly suppresses memory T-cell function. Leukemia. 2012;26:2148–51.
40. Hoek RM, Ruuls SR, Murphy CA, Wright GJ, Goddard R, Zurawski SM, et al. Down-regulation of the macrophage lineage through interaction with OX2 (CD200). Science. 2000;290:1768–71.
41. Jenmalm MC, Cherwinski H, Bowman EP, Phillips JH, Sedgwick JD. Regulation of myeloid cell function through the CD200 receptor. J Immunol. 2006;176:191–9.

References

1. Lee DW, Kochenderfer JN, Stetler-Stevenson M, Cui YK, Delbrook C, Feldman SA, Fry TJ, Orentas R, Sabatino M, Shah NN, Steinberg SM, Stroncek D, Tschernia N, Yuan C, Zhang H, Zhang L, Rosenberg SA, Wayne AS, Mackall CL. 2015. T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial. Lancet;385(9967):517-28.

2. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, Chew A, Gonzalez VE, Zheng Z, Lacey SF, Mahnke YD, Melenhorst JJ, Rheingold SR, Shen A, Teachey DT, Levine BL, June CH, Porter DL, Grupp SA. 2014. Chimeric antigen receptor T cells for sustained remissions in leukemia. N Engl J Med;371(16):1507-17.

3. Sadelain M, Brentjens R, Riviere I. 2013. The basic principles of chimeric antigen receptor design. Cancer Discov;3(4):388-98.

4. Bouchkouj N, Kasamon YL, de Claro RA, George B, Lin X, Lee S, Blumenthal GM, Bryan W, McKee AE, Pazdur R. 2019. FDA Approval Summary: Axicabtagene Ciloleucel for Relapsed or Refractory Large B-cell Lymphoma. Clin Cancer Res;25(6):1702-8.

5. O'Leary MC, Lu X, Huang Y, Lin X, Mahmood I, Przepiorka D, Gavin D, Lee S, Liu K, George B, Bryan W, Theoret MR, Pazdur R. 2019. FDA Approval Summary: Tisagenlecleucel for Treatment of Patients with Relapsed or Refractory B-cell Precursor Acute Lymphoblastic Leukemia. Clin Cancer Res;25(4):1142-6.

6. Gardner RA, Finney O, Annesley C, Brakke H, Summers C, Leger K, Bleakley M, Brown C, Mgebroff S, Kelly-Spratt KS, Hoglund V, Lindgren C, Oron AP, Li D, Riddell SR, Park JR, Jensen MC. 2017. Intent-to-treat leukemia remission by CD19 CAR T cells of defined formulation and dose in children and young adults. Blood;129(25):3322-31.

7. Maude SL, Laetsch TW, Buechner J, Rives S, Boyer M, Bittencourt H, Bader P, Verneris MR, Stefanski HE, Myers GD, Qayed M, De Moerloose B, Hiramatsu H, Schlis K, Davis KL, Martin PL, Nemecek ER, Yanik GA, Peters C, Baruchel A, Boissel N, Mechinaud F, Balduzzi A, Krueger J, June CH, Levine BL, Wood P, Taran T, Leung M, Mueller KT, Zhang Y, Sen K, Lebwohl D, Pulsipher MA, Grupp SA. 2018. Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia. N Engl J Med;378(5):439-48.

8. Seimetz D, Heller K, Richter J. 2019. Approval of First CAR-Ts: Have we Solved all Hurdles for ATMPs? Cell Med;11:2155179018822781.

9. Ruella M, Xu J, Barrett DM, Fraietta JA, Reich TJ, Ambrose DE, Klichinsky M, Shestova O, Patel PR, Kulikovskaya I, Nazimuddin F, Bhoj VG, Orlando EJ, Fry TJ, Bitter H, Maude SL, Levine BL, Nobles CL, Bushman FD, Young RM, Scholler J, Gill SI, June CH, Grupp SA, Lacey SF, Melenhorst JJ. 2018. Induction of resistance to chimeric antigen receptor T cell therapy by transduction of a single leukemic B cell. Nat Med;24(10):1499-503.

10. Orlando EJ, Han X, Tribouley C, Wood PA, Leary RJ, Riester M, Levine JE, Qayed M, Grupp SA, Boyer M, De Moerloose B, Nemecek ER, Bittencourt H, Hiramatsu H, Buechner J, Davies SM, Verneris MR, Nguyen K, Brogdon JL, Bitter H, Morrissey M, Pierog P, Pantano S, Engelman JA, Winckler W. 2018. Genetic mechanisms of target antigen loss in CAR19 therapy of acute lymphoblastic leukemia. Nat Med;24(10):1504-6.

11. Shah NN, Fry TJ. 2019. Mechanisms of resistance to CAR T cell therapy. Nat Rev Clin Oncol;16(6):372-85.

12. Tokarew N, Ogonek J, Endres S, von Bergwelt-Baildon M, Kobold S. 2019. Teaching an old dog new tricks: next-generation CAR T cells. Br J Cancer;120(1):26-37.

13. Hollyman D, Stefanski J, Przybylowski M, Bartido S, Borquez-Ojeda O, Taylor C, Yeh R, Capacio V, Olszewska M, Hosey J, Sadelain M, Brentjens RJ, Riviere I. 2009. Manufacturing validation of biologically functional T cells targeted to CD19 antigen for autologous adoptive cell therapy. J Immunother;32(2):169-80.

14. Bajgain P, Mucharla R, Wilson J, Welch D, Anurathapan U, Liang B, Lu X, Ripple K, Centanni JM, Hall C, Hsu D, Couture LA, Gupta S, Gee AP, Heslop HE, Leen AM, Rooney CM, Vera JF. 2014. Optimizing the production of suspension cells using the G-Rex "M" series. Mol Ther Methods Clin Dev;1:14015.

15. Mock U, Nickolay L, Philip B, Cheung GW, Zhan H, Johnston ICD, Kaiser AD, Peggs K, Pule M, Thrasher AJ, Qasim W. 2016. Automated manufacturing of chimeric antigen receptor T cells for adoptive immunotherapy using CliniMACS prodigy. Cytotherapy;18(8):1002-11.

16. Schneider D, Xiong Y, Wu D, Nlle V, Schmitz S, Haso W, Kaiser A, Dropulic B, Orentas RJ. 2017. A tandem CD19/CD20 CAR lentiviral vector drives on-target and off-target antigen modulation in leukemia cell lines. J Immunother Cancer;5:42.

17. Maude S, Barrett DM. 2016. Current status of chimeric antigen receptor therapy for haematological malignancies. Br J Haematol;172(1):11-22.

18. Wang X, Popplewell LL, Wagner JR, Naranjo A, Blanchard MS, Mott MR, Norris AP, Wong CW, Urak RZ, Chang WC, Khaled SK, Siddiqi T, Budde LE, Xu J, Chang B, Gidwaney N, Thomas SH, Cooper LJ, Riddell SR, Brown CE, Jensen MC, Forman SJ. 2016. Phase 1 studies of central memory-derived CD19 CAR T-cell therapy following autologous HSCT in patients with B-cell NHL. Blood;127(24):2980-90.

19. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, Almeida JR, Gostick E, Yu Z, Carpenito C, Wang E, Douek DC, Price DA, June CH, Marincola FM, Roederer M, Restifo NP. 2011. A human memory T cell subset with stem cell-like properties. Nat Med;17(10):1290-7.

20. Giavridis T, van der Stegen SJC, Eyquem J, Hamieh M, Piersigilli A, Sadelain M. 2018. CAR T cell-induced cytokine release syndrome is mediated by macrophages and abated by IL-1 blockade. Nat Med;24(6):731-8.

21. Jackson Z, Roe A, Sharma AA, Lopes F, Talla A, Kleinsorge-Block S, Zamborsky K, Schiavone J, Manjappa S, Schauner R, Lee G, Liu R, Caimi PF, Xiong Y, Krueger W, Worden A, Kadan M, Schneider D, Orentas R, Dropulic B, Sekaly RP, de Lima M, Wald DN, Reese JS. 2020. Automated Manufacture of Autologous CD19 CAR-T Cells for Treatment of Non-hodgkin Lymphoma. Front Immunol;11:1941.

22. Fernandez L, Fernandez A, Mirones I, Escudero A, Cardoso L, Vela M, Lanzarot D, de Paz R, Leivas A, Gallardo M, Marcos A, Romero AB, Martinez-Lopez J, Perez-Martinez A. 2019. GMP-Compliant Manufacturing of NKG2D CAR Memory T Cells Using CliniMACS Prodigy. Front Immunol;10:2361.

23. Grobner SN, Worst BC, Weischenfeldt J, Buchhalter I, Kleinheinz K, Rudneva VA, Johann PD, Balasubramanian GP, Segura-Wang M, Brabetz S, Bender S, Hutter B, Sturm D, Pfaff E, Hubschmann D, Zipprich G, Heinold M, Eils J, Lawerenz C, Erkek S, Lambo S, Waszak S, Blattmann C, Borkhardt A, Kuhlen M, Eggert A, Fulda S, Gessler M, Wegert J, Kappler R, Baumhoer D, Burdach S, Kirschner-Schwabe R, Kontny U, Kulozik AE, Lohmann D, Hettmer S, Eckert C, Bielack S, Nathrath M, Niemeyer C, Richter GH, Schulte J, Siebert R, Westermann F, Molenaar JJ, Vassal G, Witt H, Project IP-S, Project IM-S, Burkhardt B, Kratz CP, Witt O, van Tilburg CM, Kramm CM, Fleischhack G, Dirksen U, Rutkowski S, Fruhwald M, von Hoff K, Wolf S, Klingebiel T, Koscielniak E, Landgraf P, Koster J, Resnick AC, Zhang J, Liu Y, Zhou X, Waanders AJ, Zwijnenburg DA, Raman P, Brors B, Weber UD, Northcott PA, Pajtler KW, Kool M, Piro RM, Korbel JO, Schlesner M, Eils R, Jones DTW, Lichter P, Chavez L, Zapatka M, Pfister SM. 2018. The landscape of genomic alterations across childhood cancers. Nature;555(7696):321-7.

24. Feucht J, Kayser S, Gorodezki D, Hamieh M, Doring M, Blaeschke F, Schlegel P, Bosmuller H, Quintanilla-Fend L, Ebinger M, Lang P, Handgretinger R, Feuchtinger T. 2016. T-cell responses against CD19+ pediatric acute lymphoblastic leukemia mediated by bispecific T-cell engager (BiTE) are regulated contrarily by PD-L1 and CD80/CD86 on leukemic blasts. Oncotarget;7(47):76902-19.

25. Kong Y, Zhang J, Claxton DF, Ehmann WC, Rybka WB, Zhu L, Zeng H, Schell TD, Zheng H. 2015. PD-1(hi)TIM-3(+) T cells associate with and predict leukemia relapse in AML patients post allogeneic stem cell transplantation. Blood Cancer J;5:e330.

26. Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T, Manning S, Greenfield EA, Coyle AJ, Sobel RA, Freeman GJ, Kuchroo VK. 2002. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. Nature;415(6871):536-41.

27. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, Zheng XX, Strom TB, Kuchroo VK. 2005. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. Nat Immunol;6(12):1245-52.

28. Chiba S, Baghdadi M, Akiba H, Yoshiyama H, Kinoshita I, Dosaka-Akita H, Fujioka Y, Ohba Y, Gorman JV, Colgan JD, Hirashima M, Uede T, Takaoka A, Yagita H, Jinushi M. 2012. Tumor-infiltrating DCs suppress nucleic acid-mediated innate immune responses through interactions between the receptor TIM-3 and the alarmin HMGB1. Nat Immunol;13(9):832-42.

29. Huang YH, Zhu C, Kondo Y, Anderson AC, Gandhi A, Russell A, Dougan SK, Petersen BS, Melum E, Pertel T, Clayton KL, Raab M, Chen Q, Beauchemin N, Yazaki PJ, Pyzik M, Ostrowski MA, Glickman JN, Rudd CE, Ploegh HL, Franke A, Petsko GA, Kuchroo VK, Blumberg RS. 2015. CEACAM1 regulates TIM-3-mediated tolerance and exhaustion. Nature;517(7534):386-90.

30. Mujib S, Jones RB, Lo C, Aidarus N, Clayton K, Sakhdari A, Benko E, Kovacs C, Ostrowski MA. 2012. Antigen-independent induction of Tim-3 expression on human T cells by the common gamma-chain cytokines IL-2, IL-7, IL-15, and IL-21 is associated with proliferation and is dependent on the phosphoinositide 3-kinase pathway. J Immunol;188(8):3745-56.

31. Yang ZZ, Grote DM, Ziesmer SC, Niki T, Hirashima M, Novak AJ, Witzig TE, Ansell SM. 2012. IL-12 upregulates TIM-3 expression and induces T cell exhaustion in patients with follicular B cell non-Hodgkin lymphoma. J Clin Invest;122(4):1271-82.

32. Zhu C, Sakuishi K, Xiao S, Sun Z, Zaghouani S, Gu G, Wang C, Tan DJ, Wu C, Rangachari M, Pertel T, Jin HT, Ahmed R, Anderson AC, Kuchroo VK. 2015. An IL-27/NFIL3 signalling axis drives Tim-3 and IL-10 expression and T-cell dysfunction. Nat Commun;6:6072.

33. Wiener Z, Kohalmi B, Pocza P, Jeager J, Tolgyesi G, Toth S, Gorbe E, Papp Z, Falus A. 2007. TIM-3 is expressed in melanoma cells and is upregulated in TGF-beta stimulated mast cells. J Invest Dermatol;127(4):906-14.

34. Sabatos CA, Chakravarti S, Cha E, Schubart A, Sanchez-Fueyo A, Zheng XX, Coyle AJ, Strom TB, Freeman GJ, Kuchroo VK. 2003. Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. Nat Immunol;4(11):1102-10.

35. Sanchez-Fueyo A, Tian J, Picarella D, Domenig C, Zheng XX, Sabatos CA, Manlongat N, Bender O, Kamradt T, Kuchroo VK, Gutierrez-Ramos JC, Coyle AJ, Strom TB. 2003. Tim-3 inhibits T helper type 1-mediated auto- and alloimmune responses and promotes immunological tolerance. Nat Immunol;4(11):1093-101.

36. Jin HT, Anderson AC, Tan WG, West EE, Ha SJ, Araki K, Freeman GJ, Kuchroo VK, Ahmed R. 2010. Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. Proc Natl Acad Sci U S A;107(33):14733-8.

37. Jones RB, Ndhlovu LC, Barbour JD, Sheth PM, Jha AR, Long BR, Wong JC, Satkunarajah M, Schweneker M, Chapman JM, Gyenes G, Vali B, Hyrcza MD, Yue FY, Kovacs C, Sassi A, Loutfy M, Halpenny R, Persad D, Spotts G, Hecht FM, Chun TW, McCune JM, Kaul R, Rini JM, Nixon DF, Ostrowski MA. 2008. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. J Exp Med;205(12):2763-79.

38. Fourcade J, Sun Z, Benallaoua M, Guillaume P, Luescher IF, Sander C, Kirkwood JM, Kuchroo V, Zarour HM. 2010. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. J Exp Med;207(10):2175-86.

39. Gao X, Zhu Y, Li G, Huang H, Zhang G, Wang F, Sun J, Yang Q, Zhang X, Lu B. 2012. TIM-3 expression characterizes regulatory T cells in tumor tissues and is associated with lung cancer progression. PLoS One;7(2):e30676.

40. Anderson AC, Joller N, Kuchroo VK. 2016. Lag-3, Tim-3, and TIGIT: Co-inhibitory Receptors with Specialized Functions in Immune Regulation. Immunity;44(5):989-1004.

41. Hohtari H, Bruck O, Blom S, Turkki R, Sinisalo M, Kovanen PE, Kallioniemi O, Pellinen T, Porkka K, Mustjoki S. 2019. Immune cell constitution in bone marrow microenvironment predicts outcome in adult ALL. Leukemia;33(7):1570-82.

42. Stumpfova M, Ratner D, Desciak EB, Eliezri YD, Owens DM. 2010. The immunosuppressive surface ligand CD200 augments the metastatic capacity of squamous cell carcinoma. Cancer Res;70(7):2962-72.

43. Petermann KB, Rozenberg GI, Zedek D, Groben P, McKinnon K, Buehler C, Kim WY, Shields JM, Penland S, Bear JE, Thomas NE, Serody JS, Sharpless NE. 2007. CD200 is induced by ERK and is a potential therapeutic target in melanoma. J Clin Invest;117(12):3922-9.

44. Palumbo GA, Parrinello N, Fargione G, Cardillo K, Chiarenza A, Berretta S, Conticello C, Villari L, Di Raimondo F. 2009. CD200 expression may help in differential diagnosis between mantle cell lymphoma and B-cell chronic lymphocytic leukemia. Leuk Res;33(9):1212-6.

45. Coles SJ, Gilmour MN, Reid R, Knapper S, Burnett AK, Man S, Tonks A, Darley RL. 2015. The immunosuppressive ligands PD-L1 and CD200 are linked in AML T-cell immunosuppression: identification of a new immunotherapeutic synapse. Leukemia;29(9):1952-4.

46. Ring EK, Markert JM, Gillespie GY, Friedman GK. 2017. Checkpoint Proteins in Pediatric Brain and Extracranial Solid Tumors: Opportunities for Immunotherapy. Clin Cancer Res;23(2):342-50.

47. Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, Lee W, Yuan J, Wong P, Ho TS, Miller ML, Rekhtman N, Moreira AL, Ibrahim F, Bruggeman C, Gasmi B, Zappasodi R, Maeda Y, Sander C, Garon EB, Merghoub T, Wolchok JD, Schumacher TN, Chan TA. 2015. Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science;348(6230):124-8.

48. Park JA, Cheung NV. 2017. Limitations and opportunities for immune checkpoint inhibitors in pediatric malignancies. Cancer Treat Rev;58:22-33.

49. Kobold S, Grassmann S, Chaloupka M, Lampert C, Wenk S, Kraus F, Rapp M, Duwell P, Zeng Y, Schmollinger JC, Schnurr M, Endres S, Rothenfusser S. 2015. Impact of a New Fusion Receptor on PD-1-Mediated Immunosuppression in Adoptive T Cell Therapy. J Natl Cancer Inst;107(8).

Acknowledgements

I would like to thank

- Prof. Tobias Feuchtinger, Prof. Sebastian Kobold and Prof. Dirk Busch for supervising this thesis, their great support and helpful feedback.
- The entire Feuchtinger Lab (especially Dana Stenger, the technicians Tanja Weisser, Nicola Habjan, Nadine Stoll, the other postdocs, as well as the MD students I had the privilege to mentor).
- Prof. Alex Marson and his lab for an outstanding postdoc experience despite unexpected global challenges.
- The colleagues at Miltenyi Biotec who contributed to the first part of this thesis.
- The COALL study center for providing patient samples and follow up data.
- The healthy blood donors.
- All our collaborators and funding sources.
- My mentors and research colleagues I had the privilege to work with in the past.
- My clinical colleagues.
- My friends and family.
- The young patients who contributed to this study and were willing to donate blood for research despite just being diagnosed with leukemia. I am deeply impressed by your courage and resilience.