Ibrutinib for improved chimeric antigen receptor T-cell production for chronic lymphocytic leukemia patients

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Abstract
Chimeric antigen receptor T (CART) cells targeting CD19 have shown promising results in the treatment of chronic lymphocytic leukemia (CLL). However, efficacy seems to be inferior compared to diffuse large B-cell lymphoma or acute lymphoblastic leukemia. Impaired T-cell fitness of CLL patients may be involved in treatment failure. Less-differentiated naïve-like T cells play an important role in CART expansion and long-term persistence in vivo. These cells are sparse in CLL patients. Therefore, optimization of CART cell production protocols enriching less differentiated T cell subsets may overcome treatment resistance. The B-cell receptor inhibitor ibrutinib targeting Bruton’s tyrosine kinase (BTK) is approved for the treatment of CLL. Besides BTK, ibrutinib additionally inhibits interleukin-2-inducible T-cell kinase (ITK) which is involved in T-cell differentiation. To evaluate the effect of ibrutinib on CART cell production, peripheral blood mononuclear cells from nine healthy donors and eight CLL patients were used to generate CART cells. T-cell expansion and phenotype, expression of homing and exhaustion makers as well as functionality of CART cells were evaluated. CART cell generation in the presence of ibrutinib resulted in increased cell viability and expansion of CLL patient-derived CART cells. Furthermore, ibrutinib enriched CART cells with less-differentiated naïve-like phenotype and decreased expression of exhaustion markers including PD-1, TIM-3 and LAG-3. In addition, ibrutinib increased the cytokine release capacity of CLL patient-derived CART cells. In summary, BTK/ITK inhibition with ibrutinib during CART cell culture can improve yield and function of CLL patient-derived CART cell products.

Abbreviations: 51Cr, chromium-51; ALL, acute lymphoblastic leukemia; BTK, Bruton’s tyrosine kinase; CAR, chimeric antigen receptor; CART, chimeric antigen receptor T cells; CLL, chronic lymphocytic leukemia; CR, complete response; DLBCL, diffuse large B-cell lymphoma; FBS, fetal bovine serum; GMP, good manufacturing practice; HDs, healthy donors; IFN, interferon; IL, interleukin; ITK, interleukin-2-inducible T-cell kinase; LAG-3, lymphocyte activation gene-3; ORR, overall response rate; PBMCs, peripheral blood mononuclear cells; PD-1, programmed cell death protein 1; PE, phycoerythrin; PI3K, phosphatidylinositol 3-kinase; TCM, central memory-like T cell; TEM, effector memory-like T cell; TNF, tumor necrosis factor; TSCM, stem cell memory-like T cell.
1 | INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia. Besides chemoimmunotherapy, novel antibody and small molecule-based treatment approaches targeting the B-cell receptor pathway or antiapoptotic proteins are complementing the therapeutic armamentarium for CLL. However, CLL is still an incurable disease and progression at a certain point is inevitable.1-3 CD19-specific chimeric antigen receptor (CAR)-modified T (CART) cells have demonstrated remarkable efficacy in patients with CD19+ B-cell malignancies and led to the approval of Kymriah (Tisagenlecleucel) for the treatment of relapsed or refractory B-cell acute lymphoblastic leukemia (ALL)4 or diffuse large B-cell lymphoma (DLBCL)5 and Yescarta (Axicabtagene Ciloleucel) for the treatment of DLBCL.6 In comparison with ALL and DLBCL, the efficacy of CD19-specific CART cells in CLL seems to be lower with overall response rates (ORRs) of 50% to 70% and complete response (CR) rates of only 20% to 30%7,8 compared to DLBCL with ORRs and CRs of 82% and 54%.6 Intrinsic T-cell defects may contribute to these differences in therapeutic efficacy of CART cells.9 The differentiation status of the transfused CART cells can have a major impact on in vivo proliferative capacity and therapeutic efficacy.10-14 Less-differentiated CART cells, particularly naïve-like T cells (Tn), and stem cell memory-like T cells (T SCM) have a high capacity for engraftment and long-term persistence in vivo.15-17 Optimized CART cell production protocols enriching for these T cell subsets may improve therapeutic outcome.18 Several specific pathway inhibitors can interfere with the differentiation of T cells. For example, mTOR inhibition not only enhances the formation of CD8+ memory T cells but also augments their antitumor functions.19 Phosphatidylinositol 3-kinase (PI3K) inhibition with idelalisib can significantly improve CART cell products, particularly enrich for less-differentiated CART cells and decrease the expression of the exhaustion markers programmed cell death protein 1 (PD-1) and T-cell immunoglobulin mucin-3 (TIM-3).18 It was also reported that addition of interleukin (IL)-21 and the GSK3β inhibitor SW119 to the culture medium can lead to an enrichment of CD19-CAR-modified CD8+ T SCM with enhanced metabolic fitness and robust, long-lasting antitumor responses.16,20,21 AKT inhibitors as well as adenosine monophosphate-activated protein kinase agonists can have similar effects on CART cell production.22,23

Ibrutinib, a Bruton’s tyrosine kinase (BTK) inhibitor, showed encouraging clinical activity in patients with B-cell malignancies, particularly in CLL patients.24-26 Ibrutinib interferes with BCR signaling by inhibiting multiple targets, including BTK as well as B-lymphocyte kinase and impairs phosphorylation of downstream effectors.27,28 Ibrutinib also targets the interleukin-2-inducible T-cell kinase (ITK), can thereby deplete T helper 2 (Th2) cells and induce a shift toward Th1 cells29 or increase Th17 cell subsets.30 It was reported that CLL patients after treatment with ibrutinib can reverse the exhausted T-cell phenotype by reducing the expression of PD-1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4).30 Furthermore, pretreatment with ibrutinib before T cell collection for CART cell therapy can reverse the dysfunction of T cells and induce the variation of CART cell phenotypes. In addition, ibrutinib combined with CART therapy in preclinical in vivo models can result in high tumor killing and sustained long-term remissions.31 However, treatment of patients with ibrutinib is not always possible and other strategies for exploiting the beneficial effects on CART cell therapy may be desirable. In our study, we investigated the impact of ibrutinib supplemented to the CART cell culture medium for enhanced CART cell production and improved treatment of CLL patients.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Primary blood samples were obtained from nine healthy donors (HDs) and eight CLL patients at the University Hospital Heidelberg, Germany. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation over Ficoll density gradient and cryopreserved in frozen medium containing 90% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA) and 10% dimethyl sulfoxide (Honeywell, Morris, NJ). CD19-positive Daudi cells (Burkitt lymphoma; CVCL_0008), CD19-negative K-562 cells (chronic myeloid leukemia;
2.2 | Vector and CD19-specific CART cell generation

CART cell generation was performed with a CD19-specific third generation (CD19.CAR.CD28.CD137zeta) retroviral CAR construct as described previously. For retroviral vector generation, 293T cells were cotransfected with the specific retroviral vector plasmid PegPam3 containing gag-pol and the RDF plasmid containing the envelope. The CART cell generation protocol was adapted from our good manufacturing practice (GMP) used for our clinical CART cell trial (NCT03676504). PBMCs were activated with anti-CD3 (OKT3; Biozol, Eching, Germany) and anti-CD28 (Biozol) antibodies in 24-well plates (Corning, Corning, NY) and cultivated with culture medium (50% RPMI, 50% Clicks Medium [EHAA; Irvine Scientific, Santa Ana, CA], 10% FBS and 2 mM L-glutamine) in two different conditions: without and in presence of 1 μM ibrutinib (Selleck Chemicals, Houston, TX). Ibrutinib was freshly added at every culture medium change (every 3-4 days). The dose was defined after titration of ibrutinib on PBMCs. An amount of 1 μM was used because higher concentrations of ibrutinib were toxic to the T cells (data not shown). From Day 2, T cells were supplemented with human interleukin-7 (IL-7, R&D Systems, Minneapolis, MN) and human interleukin-15 (IL-15, R&D Systems). The use of IL-7 and IL-15 is established as the standard GMP-grade CART cell cultivation strategy at our center. Activated T cells were transduced with the CD19.CAR, CD28.CD137zeta retroviral vector on Day 3. For better comparability of the two culturing conditions, the same number of cells was always used for the start of the culture as well as the transduction of T cell cultured with or without ibrutinib. After 14 days of culture, CART cells were harvested and cryopreserved.

2.3 | Flow cytometry

Phenotypic analysis was performed by flow cytometry as described previously. Viability was determined by near-IR (Thermo Fisher Scientific) staining. The expression of the CD19 CAR was detected by a phycoerythrin (PE)-conjugated goat anti-human IgG (Dianova, Hamburg, Germany). Staining was performed with anti-CD3-PE eFluor 610, (eBioscience, San Diego, CA), anti-CD4-Alexa Fluor 700 (eBioscience), anti-CD8-PerCP (Biolegend, San Diego, CA), anti-CCR7-PE-Cy7 (eBioscience), anti-CD45RA-APC (Biolegend), anti-PD-1-Alexa Fluor 488 (Biolegend), anti-TIM-3-BV421 (Biolegend), anti-LAG-3-APC (Biolegend), anti-CD62L-eFluo 450 (eBioscience) and anti-CXCR3-Alexa Fluor 488 (Biolegend). For intracellular cytokine staining, CD19-CART cells were separately cultivated with Daudi cells and K562 cells for 6 hours. After cultivation, T cells were stained with anti-interferon (IFN)-γ-Alexa Fluor 488 (Biolegend), anti-tumor necrosis factor (TNF)-α-BV421 (BD Biosciences), CD3, CD4 and CD8 mAbs. All samples were analyzed by flow cytometry on the BD LSRII platform (BD Biosciences, Franklin Lakes, NJ). The data were analyzed by FlowJo (FlowJo, Ashland, OR). Gating strategies are shown in Figure S1.

2.4 | Cytotoxicity assay

The cytotoxic activity of transduced effector cells was evaluated using a 4-hour chromium-51 (51Cr; Hartmann Analytic, Braunschweig, Germany) release assay as described previously. Target cells (Daudi and K562 cells) were labeled with 51Cr, washed and incubated with CART cells at various effector to target (E:T) ratios (30:1, 10:1, 3:1 and 1:1). After 4 hours of incubation, the supernatants were collected, and radioactivity was detected by 1414 WinSpectral liquid scintillation counter (PerkinElmer). Specific lysis was calculated according to the following formula: % specific lysis = (51Cr release in the test well – background51Cr release)/(maximum51Cr release – background51Cr release) × 100.

2.5 | Statistical analysis

Statistical analysis was performed using Excel (Microsoft, Redmond, WA). P values were calculated either using the two-way t test or the one-way analysis of variance (ANOVA). P values <.05 were considered statistically significant. Graphs and tables were designed using Excel or Prism 6 (GraphPad Software, Inc., La Jolla, CA). If not otherwise mentioned, results are presented as mean ± SD.

3 | RESULTS

3.1 | Influence of ibrutinib on viability, expansion and transduction efficiency

Absolute cell numbers during CART cell generation were evaluated by Trypan blue staining. Cell proliferation during CART cell generation was significantly lower in CLL-derived compared to HD-derived cells (Figure 1A). Ibrutinib significantly increased cell expansion of HD-derived cells on Day 10 of CART cell generation (without vs with ibrutinib: 20.67 ± 8.82 × 10⁶ vs 25.28 ± 10.29 × 10⁶, P = .0248; Figure 1A). In addition, ibrutinib significantly increased the viability of CD3+ T cells from HDs on Day 10 (without vs with ibrutinib: 83% ± 8% vs 87% ± 7%, P = .0183) and Day 14 (without vs with ibrutinib: 84% ± 8% vs 91% ± 6%, P = .0202; Figure 1B). Furthermore, increased transduction efficiency was observed in CLL-derived cells on Day 10 and 14 (without vs with ibrutinib: 60% ± 15% vs 69% ± 12%, P = .0086 and 47% ± 14% vs 59% ± 14%, P = .0142; Figure 1C). Finally, the absolute numbers of CLL patient-derived CART cells were...
significantly higher in presence of ibrutinib on Day 10 and Day 14 of CART cell generation (without vs with ibrutinib: $1.52 \pm 1.74 \times 10^6$ vs $2.61 \pm 4.57 \times 10^6$, $P = .0138$ and $4.05 \pm 4.37 \times 10^6$ vs $7.20 \pm 6.58 \times 10^6$, $P = .0038$; Figure 1D). On Day 10 and Day 14, there was only a limited number of potentially malignant CD20-positive B cells left (Figure S2).

### 3.2 Distribution of CD4+ and CD8+ T cells

Higher numbers of CD4+ T cells and lower numbers of CD8+ T cells were observed in CLL patient-derived CART cells compared to HDs (Figure 2A,B). Cultivation of CLL and HD-derived CART cells in presence of ibrutinib did not have any influence on the distribution of CD4+ and CD8+ T cells (Figures 2A,B and S3).

### 3.3 Distribution of T cell subpopulations during CART cell production

Less-differentiated T cells, T$_{SCM}$ and T$_N$ cells, are important for CART cell proliferation as well as long-term persistence in vivo.$^{15-17}$ T$_N$ cells are defined as CCR7 and CD45RA positive.$^{11}$ In our previous studies, we observed that during the production process almost all CART cells are CD95 positive.$^{18}$ Therefore, these T$_N$ cells have a T$_{SCM}$-like phenotype. In HD-derived CART cells, ibrutinib-supplemented production enrich for T$_N$ cells on Day 10 in all CD3+ T cells (without vs with ibrutinib: $25\% \pm 11\%$ vs $40\% \pm 11\%$, $P = .0009$; Figure 3A), CD3+/CD4+ cells (without vs with ibrutinib: $17\% \pm 9\%$ vs $32\% \pm 13\%$, $P = .0016$; Figure 3B) and CD8+ cells (without vs with ibrutinib: $34\% \pm 13\%$ vs $53\% \pm 9\%$, $P = .0007$; Figure 3C) and further increased on Day 14 (CD3+ without vs with ibrutinib: $28\% \pm 12\%$ vs $45\% \pm 13\%$, $P = .0162$; CD3+/CD4+ without vs with ibrutinib: $20\% \pm 7\%$ vs $33\% \pm 13\%$, $P = .0139$; CD3+/CD8+ without vs with ibrutinib: $35\% \pm 15\%$ vs $55\% \pm 8\%$, $P = .0038$; Figure 3A-C). PBMCs from CLL patients had significantly lower proportions of T$_N$ cells (HD vs CLL: $20\% \pm 16\%$ vs $5\% \pm 3\%$, $P = .0216$) and significantly higher proportions of TEM cells (HD vs CLL: $28\% \pm 12\%$ vs $49\% \pm 12\%$, $P = .0142$) compared to HD-derived PBMCs (Figure S4). Importantly, ibrutinib-supplemented CART cell production could significantly enrich for T$_N$ cells on Day 10 (CD3+ without vs with ibrutinib: $2\% \pm 2\%$ vs $5\% \pm 3\%$, $P = .0213$; CD3+/CD4+ without vs with ibrutinib: $1\% \pm 1\%$ vs $4\% \pm 3\%$, $P = .0122$; Figure 3D,E) and Day 14 (CD3+/CD4+ without vs with ibrutinib: $2\% \pm 2\%$ vs $6\% \pm 6\%$, $P = .0449$; Figure 3E). Besides the distribution of different
T cell subsets, the absolute yield of $T_N$ CART cells is of importance. Ibrutinib significantly increased the absolute number of CD3+ $T_N$ CART cells on Day 10 (CD3+ without vs with ibrutinib: $1.69 \pm 1.16 \times 10^6$ vs $4.39 \pm 2.23 \times 10^6$, $P = .0478$; Figure 3G) and Day 14 (CD3+ without vs with ibrutinib: $4.55 \pm 1.2 \times 10^6$ vs $22.45 \pm 10.34 \times 10^6$, $P = .0488$; Figure 3G) of CART cell production. This significant increase of $T_N$ CART

**FIGURE 2** Distribution of CD4+ and CD8+ T cells. CART cells were generated with PBMCs from HDs (n = 9) and CLL patients (n = 8) and cultivated with or without 1 μM ibrutinib. CD4+ and CD8+ T cells are depicted as percentage of transduced CART cells (Day 10 and Day 14) of HDs (n = 9, A) and CLL patient cells (n = 8, B). Mean values were calculated for each group; error bars indicate SD. Statistical significance was calculated using the one-way analysis of variance and is represented as * for $P$ values <.05. CART, chimeric antigen receptor T cells; CLL, chronic lymphocytic leukemia; HDs, healthy donors; PBMCs, peripheral blood mononuclear cells [Color figure can be viewed at wileyonlinelibrary.com]

**FIGURE 3** Distribution of T cell subpopulations. CART cells were generated with PBMCs from HDs (n = 9) and CLL patients (n = 8) and cultivated with or without 1 μM ibrutinib. Distribution of Naïve-like T ($T_N$) cells (CD45RA+CCR7+), central memory-like T ($T_{CM}$) cells (CD45RA-CCR7+), effector memory-like T ($T_{EM}$) cells (CD45RA-CCR7-) and effector-like T ($T_{Eff}$) cells (CD45RA+CCR7-) of HD-derived CART cells in CD3+ (A), CD3+/CD4+ (B) and CD3+/CD8+ (C) subsets as well as CLL patient-derived CART cells in CD3+ (D), CD3+/CD4+ (E) and CD3+/CD8+ (F) subsets on Day 10 and 14 of CART cell generation. Absolute numbers of CAR+ $T_N$ cells generated with HD-derived (G) and CLL patient-derived (H) PBMCs in CD3+, CD3+/CD4+ and CD3+/CD8+ subsets on Day 10 and 14 of CART cell generation. Mean values were calculated for each group; error bars indicate SD. Statistical significance was calculated using the two-way t test and is represented as * for $P$ values <.05. CART, chimeric antigen receptor T cells; CLL, chronic lymphocytic leukemia; HDs, healthy donors; PBMCs, peripheral blood mononuclear cells [Color figure can be viewed at wileyonlinelibrary.com]
cells was seen in the CD4+ Th cell (Day 10 without vs with ibrutinib: 0.64 ± 0.25 × 10^6 vs 2.2 ± 1.02 × 10^6, P = .0447, Day 14 without vs with ibrutinib: 1.72 ± 0.59 × 10^6 vs 3.98 ± 1.71 × 10^6, P = .0412; Figure 3G) as well as CD8+ T cell (Day 10 without vs with ibrutinib: 0.99 ± 0.94 × 10^6 vs 2.22 ± 1.51 × 10^6, P = .0393, Day 14 without vs with ibrutinib: 2.83 ± 1.49 × 10^6 vs 14.61 ± 7.95 × 10^6, P = .0357; Figure 3G) fraction. Importantly, the increased production of CD3+ TCU CART cells was also seen in productions with PBMCs from CLL patients (Day 10 without vs with ibrutinib: 2.32 ± 1.87 × 10^4 vs 9.76 ± 0.06 × 10^4, P = .0049, Day 14 without vs with ibrutinib: 6.81 ± 9.18 × 10^4 vs 25.21 ± 30.32 × 10^4, P = .0439; Figure 3H). Again, the increased yield of TCU CART cells was seen in the CD4+ (Day 10 without vs with ibrutinib: 0.99 ± 1.03 × 10^4 vs 2.39 ± 0.05 × 10^4, P = .0192, Day 14 without vs with ibrutinib: 3.14 ± 5.87 × 10^4 vs 6.66 ± 9.37 × 10^4, P = .0403; Figure 3H) T-cell fractions.

3.4 Analysis of homing and exhaustion markers

Expression of CXCR3 and CD62L was determined to evaluate T-cell homing capacities of CART cells generated in presence of ibrutinib. High surface levels of CXCR3 are linked to peripheral tissue and high surface levels of CD62L to lymphoid tissue homing.36 Especially the high expression of CD62L may mediate preferred homing of CART cells into the tumor sites of lymphoid malignancies. No differences in CXCR3 expression were seen between both production strategies in HD and CLL patient-derived CART cells (Figure 4A). However, significantly higher CD62L expression was observed after cultivation with ibrutinib in CLL patient-derived CART cells on Day 14 (without vs with ibrutinib: 58% ± 17% vs 66% ± 10%, P = .0455; Figure 4B). PD-1, TIM-3 and lymphocyte activation gene-3 (LAG-3) were evaluated to determine exhaustion of CART cells. Ibrutinib-supplemented CART cell production significantly reduced PD-1 expression of CLL patient-derived CART cells (Day 10 without vs with ibrutinib: 59% ± 24% vs 52% ± 20%, P = .0063, Day 14: 44% ± 22% vs 32% ± 16%, P = .0149; Figure 4C). In addition, TIM-3 expression was significantly lower using the ibrutinib-based production strategy in HD-derived CART cells (Day 10 without vs with ibrutinib: 90% ± 10% vs 74% ± 20%, P = .0224, Day 14: 73% ± 20% vs 56% ± 28%, P = .0425; Figure 4D) as well as CLL patient-derived CART cells (Day 10 without vs with ibrutinib: 96% ± 3% vs 82% ± 17%, P = .0305, Day 14: 86% ± 11% vs 72% ± 20%, P = .0066; Figure 4D). Furthermore, ibrutinib significantly decreased LAG-3 expression in HD-derived CART cells on Day 10 (without vs with ibrutinib: 37% ± 16% vs 18% ± 10%, P = .0178) as well as CLL patient-derived CART cells on Day 10 (69% ± 21% vs 49% ± 19%, P = .0002) and Day 14 (34% ± 17% vs 22% ± 14%, P = .0242) of CART cell production (Figure 4E). Moreover, coexpression analysis of exhaustion markers revealed significantly reduced coexpression of PD-1/Tim-3 (Day 10 without vs with
ibrutinib: 53% ± 24% vs 40% ± 22%, P = .0008, Day 14: 34% ± 23% vs 21% ± 16%, P = .0099; Figure 4F), PD-1/LAG-3 (Day 10 without vs with ibrutinib: 37% ± 25% vs 22% ± 15%, P = .0088, Day 14: 14% ± 13% vs 6% ± 6%, P = .0488; Figure 4G) as well as PD-1/TIM-3/LAG-3 (Day 10 without vs with ibrutinib: 30% ± 14% vs 16% ± 9%, P = .0408, Day 14: 10% ± 5% vs 3% ± 2%, P = .0479; Figure 4H) in CLL patient-derived CART cells generated in presence of ibrutinib.

3.5 | Evaluation of cytokine production

Cytokine production of CART cells was evaluated by intracellular FACS staining after stimulation with CD19-positive Daudi cells. CART cells from CLL patients that were generated in presence of ibrutinib displayed significantly higher intracellular production of TNF-α (without vs with ibrutinib: 47% ± 11% vs 60% ± 18%, P = .0392; Figure 5A), particularly in the CD8+ CART cell subpopulation (without vs with ibrutinib: 30% ± 14% vs 16% ± 9%, P = .0408, Day 14: 10% ± 5% vs 3% ± 2%, P = .0479; Figure 4H) in CLL patient-derived CART cells generated in presence of ibrutinib.

3.6 | Evaluation of antigen-specific cytotoxicity

Functional analysis for CART cell-mediated cytotoxicity was performed by 51Cr release assay. Ibrutinib for CART cell generation did not have significant effects on in vitro cytotoxic capacities of HD and CLL patient-derived CART cells against CD19-positive Daudi cells (10:1 ratio without vs with ibrutinib: 52% ± 15% vs 42% ± 12%, P = .3 and 35% ± 18% vs 40% ± 13%, P = .5; Figure 5D,E). No relevant unspecific cytotoxicity against CD19-negative K-562 cells was observed (Figure 5F). A significant difference in cytotoxic capacity was seen between HD and CLL patient-derived CART cells generated in absence of ibrutinib (3:1 ratio HD vs CLL: 42% ± 11% vs 20% ± 12%, P = .0421, Figure 5G). This may reflect the T-cell defect and the reduced effector function of CLL-derived T cells.

4 | DISCUSSION

CD19-CART cell therapy is a promising treatment approach for patients with CD19-positive B-cell malignancies. However, efficacy is clearly lower for CLL patients compared to ALL and DLBCL. Therefore, novel strategies are needed to improve the clinical benefit using CART cells for CLL patients. Optimization of the CART production process may be a simple but effective approach to improve efficacy of CART cells in CLL patients. In our study, we evaluated the effects of
ibrutinib-supplemented CART cell production with a particular focus on CLL patient-derived CART cells.

Intrinsic defects of CLL patient-derived T cells impair both, the feasibility of CART cell generation as well as in vivo efficacy.\(^7,^8,^{18,33}\) It was reported that CLL patients pretreated with \(>5\) cycles of ibrutinib therapy can recover the T-cell defects leading to effective ex vivo expansion of their CART cells with potentially enhanced in vivo functionality.\(^9\) However, pretreatment of CLL patients with ibrutinib is not always possible: side effects including cardiotoxicity and bleedings as well as treatment resistance may prohibit prolonged application. In the current study, ibrutinib supplemented to the CART cell production process could not only achieve significantly higher expansion of T cells but also lead to increased viability as well as transduction efficiency and thereby achieve significantly higher CART cell yields from CLL patients.

CLL patients tend to have higher CD4:CD8 T cell ratios when compared to HDs. A more balanced CD4:CD8 ratio might be beneficial for efficient CART cell therapy.\(^{17}\) In our study, ibrutinib supplemented to CART cell culture medium could not overcome this limitation in CLL patients. This stands in contrast to our previous findings that PI3K inhibition with idelalisib can lead to potentially more favorable CD4:CD8 ratios in CLL patients.\(^{18}\) This indicates that different pathways may be targeted by ibrutinib and idelalisib for optimized CART cell production.

Less-differentiated T cells, particularly TN and TSCM, are important for CART cell engraftment and in vivo persistence as well as efficient tumor eradication.\(^{14-16}\) Differentiation of primed TN cells can be suppressed by certain cytokines, or by small molecules targeting key metabolic and developmental pathways.\(^{11}\) For example, we previously reported inhibition of the PI3K/AKT/mTOR pathway with the clinically approved PI3K\(\delta\) inhibitor idelalisib can mediate CART cells with a TN cell phenotype.\(^{18}\) In the current study, we focused on the BTK inhibitor ibrutinib that is also in clinical use for the treatment of CLL. Ex vivo supplementation of ibrutinib to the culture medium significantly enriched less-differentiated CART cells with up to four times more CART cells with TN cell phenotype in the final cell product. This effect was not only seen in CART cells generated from CLL patient-derived PBMCs but also in CART cells from HDs. This indicates that ex vivo CLL cell elimination alone cannot explain the beneficial effects of ibrutinib for the CART cell production process.

More differentiated T cells such as TEM and TEff cells rather prefer homing into the peripheral tissue.\(^{37,38}\) In contrast, less-differentiated T cells, including TN, TSCM and TCM cells, tend to migrate more into the lymphoid tissue.\(^{37,39}\) In the current study, CART cell generation in presence of ibrutinib did not influence the expression of CXCR3 that is mediating trafficking into the peripheral tissue.\(^{40}\) In contrast, significantly higher expression of the lymphoid homing marker CD62L was observed in CART cells generated from CLL patients in presence of ibrutinib. CD62L expression was associated with enhanced antitumor activity in preclinical models of adoptive cell therapy\(^{14,17}\) and ibrutinib-based CART cell production may be beneficial for lymphoid malignancies with higher migration directly to the tumor site. Considering the high amounts of CXCR3 and CD62L positive cells, CART cells coexpressing both markers may have the ability to reach the lymphoid tissue as well as extranodal sites.\(^31\)

PD-1 is a key immune-checkpoint receptor expressed on activated T cells. It is a negative regulator of T-cell immune response, inhibits T-cell proliferation as well as cytokine production and reduces T-cell survival.\(^{52,42}\) Importantly, CLL patient-derived T cells were reported to be impaired with high PD-1 expression.\(^{44,45}\) LAG-3 and TIM-3 can also negatively impact T-cell function and induce cell death.\(^{46,47}\) Reduced expression of these negative regulators of T cells may increase immune activation and decrease CART cell exhaustion.\(^{48}\) We observed that ibrutinib-supplemented CART cell generation significantly reduced the expression of these exhaustion markers, especially on CLL patient-derived CART cells. LAG-3 and PD-1 coexpression was reported to characterize highly dysfunctional and exhausted T cells.\(^{49,50}\) Ibrutinib added to the culture medium could significantly decrease the coexpression of PD-1 and LAG-3, PD-1 and TIM-3 as well as the expression of all three markers on CLL patient-derived T cells. This can reflect a reduced activation of T cells in the ibrutinib-supplemented culture condition and does not directly imply less-exhausted CART cells. However, CART cells with reduced expression of exhaustion markers may also be beneficial for efficient tumor eradication, particularly in CLL patients.

CLL patient-derived CART cells generated in presence of ibrutinib displayed significantly higher production of TNF-\(\alpha\) and IFN-\(\gamma\), especially in CD8+ CART cells. In contrast, there was no difference in lytic activity in the \(^{51}\)Cr release assay between the two different production strategies. It is important to state that CLL-derived CART cells generated in both culturing conditions do not seem to be terminally exhausted as they were capable of cell expansion, cytokine production as well as target cells elimination. Currently it is unclear if differences in ex vivo cytokine production and lytic activity of CART cells can be translated into in vivo efficacy.

Optimized CART cell production protocols are already in clinical evaluation: For example, bb21217, a B-cell maturation antigen (BCMA)-targeted CART cell product, is generated in presence of a PI3K inhibitor leading to an enrichment of less-differentiated CART cells with potentially increased antitumor activity.\(^{51}\) The current study provides, to our knowledge, the first proof of concept that ibrutinib-based CART cell production may be beneficial for efficient CART cell products in CLL patients. Different pathways are probably involved in ibrutinib and PI3K-based optimization of CART cell production. Additional improvements may be achieved by the combination of both strategies with the benefits of a more balanced CD4:CD8 distribution from PI3K inhibition and the higher expansion benefits of less-differentiated phenotypes as well as increased CD62L expression achieved with ibrutinib in CLL patient-derived CART cells. However, the proportions of CLL-derived CART cells with less-differentiated phenotypes are in the one-digit percentage ranges and there is still room for improvement. For example, AKT inhibition may be a promising option to further enrich for CART cells with less-differentiated phenotypes.\(^{52,53}\)

Previous reports by Fraietta et al demonstrated improved ex vivo expansion, decreased PD1 expression as well as improved IFN-\(\gamma\) production of T cells from CLL patients who received \(>5\) cycles of
ibrutinib in vivo. In addition, they reported that ex vivo ibrutinib exposure did neither alter T-cell proliferation nor transduction efficiency. Our current study confirms these observations in our setup with ibrutinib supplemented ex vivo to the CART cell culture medium: similar effects including higher CART cell numbers, reduced PD1 expression as well as improved IFN-γ production of CART cells were achieved. Furthermore, we identified that ex vivo ibrutinib can even increase transduction efficiency as well as CART cell yields.

In conclusion, our study provides evidence that BTK/ITK inhibition with ibrutinib during CART cell generation may improve CLL patient-derived CART cell products and may have the potential to enhance CART cell function. Ibrutinib-supplemented CART cell production leads to increased CART cell yields as well as enriches for less-differentiated T cells with lower expression of exhaustion markers and could be an option to further improve clinical outcome of CLL patients. Further analyses including in vivo validation of the findings are necessary to prove this concept.

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CONFLICT OF INTEREST
A. S.: travel grants, Jazz Pharmaceuticals, Hexal; research grants, Therakos/Mallinckrodt; cofounder and part-time employee, TolerogenixX Ltd. C. M.-T.: financial support, Pfizer, Daichi Sankyo, Janssen, Bayer, BioLineRx, Deutsche Forschungsgesellschaft, Deutsche Krebshilfe, BMBF, Wilhelm-Sander-Stiftung, Jose-Carreras-Stiftung, L. S.: employment, Takeda. M.-L. S.: Honoraria, Kite/Gilead. M. S.: research funding, Apogenix, Hexal, Novartis; travel grants, Hexal, Kite/Gilead, Novartis; honoraria, Kite/Gilead, MSD; cofounder and shareholder, TolerogenixX Ltd. All other authors: no relevant conflicts of interest.

ETHICS STATEMENT
Sample collection and analysis were approved by the Ethics Committee of the University of Heidelberg (S-254/2016) and written informed consent was obtained from all patients.

DATA AVAILABILITY STATEMENT
Data can be made available upon reasonable request.

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SUPPORTING INFORMATION
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