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Selective BTK inhibition improves bendamustine therapy response and normalizes immune effector functions in chronic lymphocytic leukemia

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The Bruton's tyrosine kinase (BTK) inhibitor ibrutinib has been shown to be highly effective in patients with chronic lymphocytic leukemia (CLL) and is approved for CLL treatment. Unfortunately, resistance and intolerance to ibrutinib has been observed in several studies, opening the door for more specific BTK inhibitors. CC-292 (spebrutinib) is a BTK inhibitor with increased specificity for BTK and less inhibition of other kinases. Our *in vitro* studies showed that CC-292 potently inhibited B-cell receptor signaling, activation, proliferation and chemotaxis of CLL cells. In *in vivo* studies using the adoptive transfer TCL1 mouse model of CLL, CC-292 reduced tumor load and normalized tumor-associated expansion of T cells and monocytes, while not affecting T cell function. Importantly, the combination of CC-292 and bendamustine impaired CLL cell proliferation *in vivo* and enhanced the control of CLL progression. Our results demonstrate that CC-292 is a specific BTK inhibitor with promising performance in combination with bendamustine in CLL. Further clinical trials are warranted to investigate the therapeutic efficacy of this combination regimen.

Introduction

The B-cell receptor (BCR) signaling is a pivotal pathway in the pathogenesis and progression of chronic lymphocytic leukemia (CLL) and is critical for B-cell proliferation, survival and migration. Upon BCR activation, Bruton's tyrosine kinase (BTK) translocates to the membrane and becomes phosphorylated by SRC kinases, leading to BTK autophosphorylation at Y223, which in turn induces the phosphorylation and activation of PLCγ2. This triggers calcium mobilization and propagation of BCR signaling.[1–3]

The standard of care for patients with symptomatic CLL includes fludarabine plus cyclophosphamide and rituximab.[4] However, bendamustine is a well-tolerated and less toxic agent that has emerged as a more feasible therapy for elder and unfit CLL patients in combination with rituximab (BR regimen).[5] Bendamustine is a bifunctional agent that combines the properties of an alkylator and a purine analogue. Although bendamustine engages cell death through both dependent and independent p53 pathways,[6–8] patients with 17p deletions have shown to poorly respond to BR.[9,10] In addition, signals transmitted by the microenvironment play a key role in promoting cell survival and chemoresistance of CLL cells to bendamustine.[11] Ongoing clinical trials explored the effects of

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What's new?

B-cell receptor (BCR) signaling plays a pivotal role in chronic lymphocytic leukemia (CLL). BTK inhibitor ibrutinib has been shown to be highly effective in patients, but resistance and intolerance have also been observed. Here, the authors demonstrate that the more specific CC-292 disrupts BCR signaling and inhibits tumor cell activation, proliferation, and chemotaxis *in vitro*. In mice, CC-292 reduces tumor load and normalizes tumor-associated expansion of T cells and monocytes while not affecting T cell function. Combination of CC-292 and bendamustine impairs CLL cell proliferation and normalizes immune cell composition, which overcomes microenvironment-mediated chemoresistance and enhances control of CLL progression.

bendamustine combinations with new generation monoclonal antibodies and novel targeted agents.[12]

Ibrutinib, the first-in-class BTK inhibitor, is approved for the treatment of untreated CLL patients,[11,13] patients with relapsed or refractory disease,[14,15] and patients with 17p deletions.[16] However, most patients do not experience a complete response and a subset of patients develops resistance, mainly through mutations in *BTK* or *PLCγ2*.[17] CLL patients who relapse or progress while receiving this agent have a very poor outcome.[18] On the other hand, alternative targets of ibrutinib (ITK, EGFR and TEC) may account for some adverse effects that lead to therapy discontinuation.[19] In this regard, a more specific BTK inhibitor may have therapeutic benefit.

Spebrutinib (CC-292) is a highly selective oral BTK inhibitor that covalently and irreversibly binds the same Cys481 in BTK as ibrutinib, with increased specificity for BTK and less inhibition of other kinases.[20] In a phase I study, spebrutinib was well tolerated and resulted in nodal responses, although durability of response was inferior to that of ibrutinib.[21]

Herein, we aimed to evaluate the antitumor potential of CC-292 in *in vitro* and *in vivo* CLL models and to assess the potential combination of CC-292 with bendamustine in the TCL1 adoptive transfer (TCL1 AT) mouse model of CLL.

Materials and Methods

Primary CLL cells

Primary cells from 53 patients diagnosed with CLL according to the World Health Organization criteria[22] were used in our study. Clinical and biological data of each patient are detailed in Table S1, Supporting Information. Cells were isolated from peripheral blood (PB) or bone marrow (BM) samples by Ficoll-Paque sedimentation (GE-Healthcare, Chicago, IL) and from lymph nodes (LN) by squirting RPMI-1640 medium (Life Technologies, Paisley, UK) using a fine needle. Samples were cryopreserved and stored within the Hematopathology collection of our institution registered at the Biobank from Hospital Clínic-IDIBAPS (R121004-094). The ethical approval for this project including the informed consent of the patients was granted, after the guidelines of the Hospital Clínic Ethics Committee and the Declaration of Helsinki. Thawed cells were cultured in fresh RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Life technologies), 2 mM glutamine and 50 μg/mL penicillin–streptomycin (Life technologies) and cultured in a humidified atmosphere at 37 °C containing 5% carbon dioxide.

Drugs

CC-292 and CNX-652 (CC-292 for *in vivo* usage) were kindly provided by Celgene Corporation (San Diego, CA). CC-292 was dissolved in DMSO and used at 1μM in all *in vitro* experiments. CNX-652 was prepared fresh every 6 days in warm DMSO (5%), Solutol HS 15 (15%) and PBS (80%), sonicated for 10 min, and stored at 4 °C. For the *in vitro* experiments, bendamustine was provided by Mundipharma (Cambridge, UK), whereas for the *in vivo* study, bendamustine (Levact®) was purchased from the Hospital Clinic pharmacy and dissolved in physiological serum before usage.

Analysis of cytotoxicity

Primary samples from 41 CLL patients with ≥85% tumor cells were incubated for 48 and 72 h with 1 μM CC-292. Cell viability was quantified by double staining with Annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) (eBiosciences, Santa Clara, CA). For the comparative analysis of response in the PB, BM and LN compartments, cells were further stained with phycoerythrin (PE)-anti-CD19 (BD biosciences, San Jose, CA). For the comparison between response in PB CLL cells and normal B and T lymphocytes from healthy donors, PE-Cy5-conjugated anti-CD3 (BD biosciences) was added to the anti-CD19/Annexin-V/PI panel. Labeled samples were analyzed on an Attune focusing acoustic cytometer (Life Technologies). Cytotoxicity (mean ± s.e.m.) was determined from the percentage of viable cells, which were defined as Annexin-V/PI double negative cells. Depletion of intracellular ATP levels in CLL cells was determined by using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI).

BCR signaling pathway

Primary samples with ≥94% CLL cells were washed twice and serum-starved for 1 h in FBS-free RPMI-1640 at 10^7 cells/mL. CC-292 (1 μM) was added for 1.5 additional hours, and a 2-min BCR stimulation was performed with 10 μg/mL αIgM (Southern Biotech, Birmingham, AL) and 3.3 mM hydrogen peroxide (H_2O_2) for inhibition of phosphatases. Immediately afterwards, cells were fixed in 1% paraformaldehyde for 1 h at 4 °C, washed with PBS 1× and permeabilized overnight with 500 μL ethanol 70% at −20 °C. Cells were then washed and

stained with PE-conjugated anti-BTK (pY223) and FITC-conjugated anti-PLCγ2 (pY759) (BD biosciences). Median fluorescence intensity (MFI) of 10000 lymphocytes was analyzed on an Attune cytometer and the percentage of the activation state of both BCR kinases was calculated relative to the stimulated control.

Chemotaxis assay

CLL cells (10^7 cells/mL) were washed and serum-starved for 1 h in FBS-free RPMI-1640. CC-292 was added for 1.5 additional hours, followed by 15-min BCR stimulation with 10 μg/mL αIgM. Cells were then diluted to 5×10^6 cells/mL with 0.5% bovine serum albumin (BSA; Sigma, Saint Louis, MO) in RPMI-1640. One hundred microliters (5×10^5 cells) were added to the top chamber of a transwell culture polycarbonate insert with 6.5-mm diameter and 5 μm of pore size (Corning, Corning, NY). Inserts had been previously coated with VCAM-1 (Peprotech, London, UK) overnight, washed with PBS, and transferred to wells containing 600 μL of RPMI with or without 200 ng/mL of human recombinant CXCL12 (Peprotech). After 3 h of incubation, 100 μL were collected in triplicate from each lower chamber and viable cells counted on an Attune cytometer for 12 s under a constant flow rate of 500 μL/min.

In vitro B-CLL proliferation assay

CLL primary cells (10^7 cells) were labeled with 0.5 μM carboxyfluoresceinsuccinimidyl ester (CFSE) (Thermo Fisher Scientific, Waltham, MA), seeded in 96-well plates (Falcon, Corning, NY) at 10^5 cells/200 μL, and cultured for 6 and 9 days in an enriched RPMI-1640 medium used for long-term cultures,[23] supplemented with 15 ng/mL recombinant human IL-15 (R&D systems, Minneapolis, MN) to sustain survival. To induce cell proliferation, 0.2 μM CpG DNA TLR-9 ligand (ODN-2006; Invivogen, San Diego, CA) was added, together with CC-292 1 μM in indicated conditions. The percentage of divided cells was determined as the percentage of $CD19^+$ (PE) / Annexin-V^- (Pacific Blue) cells showing a decrease in CFSE staining on flow cytometry.

Coculture experiments

Coculture experiments of CLL cells with the human bone marrow-derived mesenchymal cell line-HS-5 (American Type Culture Collection)(ATCC®, CRL11882™) and the human follicular dendritic cell-like cell line HK, kindly provided by Dr. Y.S. Choi, were performed as previously described.[11] For the combinatorial experiments of 1 μM CC-292 and 25 μM bendamustine, CLL cells were treated for 48 h and cell viability was quantified by means of Annexin-V/PI staining. For the analysis of drug interference on tumor cell activation, cells were incubated with or without 1 μM CC-292 for 24 h and stained against CD19-FITC and CD69-PE (BD biosciences).

TCL1 adoptive transfer (AT) mouse model

Eμ-TCL1 (TCL1) mice on C57BL/6 background were kindly provided by Dr. Carlo Croce (Ohio State University). In this model, the overexpression of TCL1 in B cells under the VH-promoter-IgH-Eμ-enhancer drives a clonal expansion of $CD5^+$ B cells, representing an aggressive form of CLL.[24] For treatment studies, adoptive transfer of TCL1 tumors were performed in C57BL/6 WT mice as described before.[25] Briefly, 10^6 splenocytes with more than 95% of viable $CD19^+CD5^+$ cells from leukemic TCL1 mice were transplanted in 3-month-old female C57BL/6N wild-type mice (Charles River Laboratories, London, UK) *via* tail vein injection. Mice were housed in pathogen-free conditions, closely monitored for signs of illness. All experiments were performed according to the University of Barcelona animal experimental ethics committee guidelines. When PB tumor load (TL) reached mean values of 50% of $CD19^+$ $CD5^+$ (out of total $CD45^+$ cells), animals were randomized into 4 groups (Vehicle, CC-292, bendamustine and Combination) with equal mean and standard deviation of TL percentage values. Fifteen milligram/kilogram CC-292 were administered twice daily *via* oral gavage, whereas 25 mg/kg bendamustine were administered intravenously once weekly. Mice were euthanized after 11 days of treatment and single cell suspensions were obtained from BM, inguinal LN and spleen.

Analysis of cell suspensions from mice

Cell suspensions from the bone marrow were flushed from one femur with 5 mL of PBS/5% FBS. Cell suspensions from one inguinal lymph node and from the spleen were obtained by pressing with a 3 mL syringe plunger in 5 mL of PBS/5% FBS. Cell suspensions were after homogenized and filtrated through 70 μm nylon sieves (BD Falcon™). Erythrocytes were lysed using ACK buffer (Cultek, Berks, UK). PB was drawn weekly and hematological counts were obtained in an automated V-Sight hemocytometer (Menarini diagnostics, Firenze, IT).

After preparations of single-cell suspensions, cells were incubated with recommended dilutions of antibodies against cell surface proteins in PBS containing 0.1% fixable viability dye (eBiosciences, Frankfurt am Main, Germany) for 30 min at 4 °C. Cells were fixed using IC fixation buffer (eBioscience), washed and stored at 4 °C in the dark until analyzed by flow cytometry. For labeling of cells in whole blood, 50–100 μL of PB were stained with antibodies specific for surface molecules for 30 min at 4 °C, followed by incubation for 10 min with 2 mL of 1× BD FACS™ lysing solution (BD Biosciences, Heidelberg, Germany) to remove erythrocytes. After centrifugation, supernatants were carefully aspirated and pelleted cells were resuspended in 150 μL of 1× BD FACS™ lysing solution. A list of the antibodies used is showed in Table S2, Supporting Information.

For Ki-67 staining, spleen cells were fixed after surface staining with Foxp3 fixation/permeabilization buffer (eBiosciences) for 30 min at room temperature (RT), permeabilized and stained with anti-Ki-67 (eBiosciences) or the respective

isotype controls in 1× permeabilization buffer for 30 min. After washing, cells were resuspended in 1× permeabilization buffer and data was acquired. Data acquisition was performed on a BD FACSCanto II, Fortessa, or BD LSRII flow cytometer (BD Biosciences). MFI was normalized by subtracting the MFI of the respective fluorescence-minus-one (FMO) control. Data analysis was performed using FlowJo X 10.0.7 software (FlowJo, Ashland, OR).

$CD8^+$ T-cell functional assays

Cytokine release and degranulation capacity of $CD8^+$ T cells were assayed as previously described[26] with minor modifications. Cells were resuspended in DMEM supplemented with 10% FCS, 10 mM HEPES, 1 mM Sodium pyruvate, β-Mercaptoethanol 100 U/mL penicillin and 100 μg/mL streptomycin, and seeded at 3×10^6 cells/200 μL. Cells were stimulated with cell stimulation cocktail in the presence of protein transport inhibitor cocktail (both from eBioscience) for 6 h at 37 °C/5% CO_2. Degranulation capacity of T-cells was measured by adding fluorochrome-conjugated CD107a antibody (eBioscience) to the culture, as previously described.[27] Afterwards, cells were washed, stained for surface proteins and fixed using IC fixation buffer (eBioscience). After washing with 1× permeabilization buffer, cells were stained with fluorescently labeled antibodies against intracellular proteins, washed and analyzed by flow cytometry.

Statistical analysis

Data analysis was performed using Graphpad Prism 6.01 software (GraphPad Software, La Jolla, CA). All results are expressed as mean ± s.e.m. For *in vitro* studies, a parametric or nonparametric paired *t*-test was performed according to sample size. For *in vivo* studies, pairwise comparisons were performed using unpaired *t*-test with Welch's correction to account for unequal variances. Statistical significance was considered when p value<0.05.

Results

CC-292 is an effective BTK inhibitor that impairs CLL activation and CXCL12-induced chemotaxis

We analyzed the ability of CC-292 to interfere with BCR signaling in CLL cells, by measuring phosphorylation of BTK at position Y223 and PLCγ2 at position Y759, which is direct downstream target of BTK. Upon αIgM-stimulation, we observed a significant increase in BTK ($p < 0.0001$) and PLCγ2 phosphorylation ($p < 0.0001$; shift from blue line to black line) that was significantly inhibited ($p = 0.0012$ for p-BTK and $p = 0.0038$ for p-PLCγ2) by incubation with CC-292 (red line) (Fig. 1*a*).

CC-292 treatment for 24 h reduced the levels of the early B cell activation marker CD69 on CLL cells ($p = 0.03$; Fig. 1*b*) and, importantly, it was able to overcome significant upregulation of CD69 in cocultures of CLL cells with stromal HS-5 cells or dendritic HK cells (Fig. 1*b*). We also analyzed the effect of CC-292 on VCAM-1-mediated adhesion and concomitant migration of CLL cells triggered by CXCL12. Chemotaxis of CLL cells was significantly enhanced after engagement of BCR with αIgM ($p = 0.0002$) and CC-292 was fully capable of decreasing CLL chemotaxis at the level of nonstimulated cells ($p = 0.001$; Fig. 1*c*). Together, these data indicate that CC-292 disrupts BCR signaling and, consequently, impairs activation and chemotaxis of CLL cells.

CC-292 induces modest apoptosis in CLL cells

Next, we investigated the cytotoxic effect of CC-292 *in vitro* in primary cells from 43 CLL patients (Table S3, Supporting Information). CC-292 modestly but significantly decreased CLL cell viability by 10.2% ± 1.5 and 17.4% ± 1.9 after 48 h and 72 h of incubation, respectively (both $p < 0.0001$; Fig. 2*a*). CC-292 also significantly reduced intracellular ATP levels by 20% ± 3.3 at 48 h and by 32% ± 3.5 at 72 h (both $p < 0.0001$; Supporting Information Fig. S1*a*). CC-292 cytotoxic effect was lower than ibrutinib (Supporting Information Fig. S2*a* and S2*b*) and, importantly, T and B lymphocytes from healthy donors were significantly less sensitive to CC-292 compared to CLL cells (T-cells: $p < 0.029$; B cells: $p < 0.045$; Fig. 2*b*). We observed that cases belonging to the unmutated IGHV CLL subgroup (IGHV-UM) were more responsive to CC-292 compared to the IGHV mutated subgroup (IGHV-M) (IGHV-UM: 20.4% ± 2.6 cytotoxicity *versus* IGHV-M: 11.3% ± 2.1; $p = 0.026$; Fig. 2*c*).

CLL cell proliferation is potently blocked by CC-292

To evaluate CC-292 capability to inhibit CLL cell proliferation, CFSE-labeled primary CLL cells were induced to proliferate by incubating them with a medium containing CpG oligodeoxynucleotide, which triggers growth and cell division in the proliferative centers of CLL patients, and the inflammation-linked cytokine IL-15, which is constitutively produced by stromal cells.[23] This medium significantly increased mean percentage of $CFSE^{low}$ viable B cells from 2.6% to 41.8% after 6 days of incubation, and from 3.0% to 50.9% after 9 days (both $p < 0.0001$; Supporting Information Table S4). A representative case is showed in Figure 2*d*. This increase was significantly reduced by CC-292 treatment to 5.4% (8-fold decrease) at 6 days, and to 6.9% (7-fold decrease) at 9 days (both $p < 0.0001$; Fig. 2*e*). Moreover, this effect was comparable to the effect observed with ibrutinib (Supporting Information Fig. S3*a*) and independent of the mutational status of the IGHV genes (Supporting Information Fig. S3*b*). CC-292 also exerted an anti-proliferative effect in tumor cells isolated from the BM of CLL patients, both at 6 and 9 days (Fig. 2*f*).

CC-292 and bendamustine cooperate to overcome stromal protection of CLL cells

Based on our previous observation that high expression of CD69 is an independent marker of resistance to bendamustine in CLL[11] and our results showing that CC-292 prevents significant upregulation of CD69 on CLL cells by the microenvironment, we tested the combination of CC-292 with bendamustine

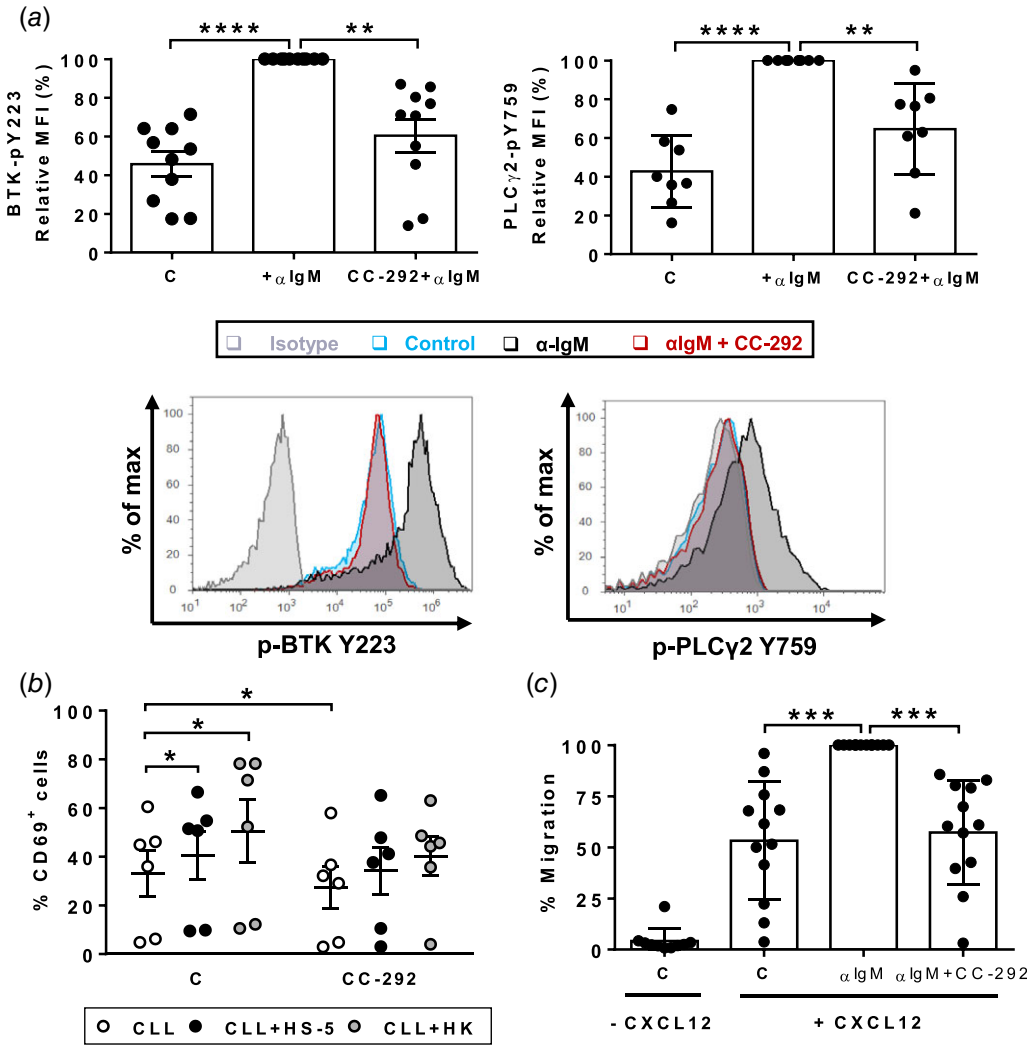

Figure 1. CC-292 inhibits BCR signaling, overcomes stroma-induced activation, and impairs chemotaxis of CLL cells. (*a*) Mean fluorescence intensity (MFI) of pY223 BTK ($n = 10$) and pY759 PLCγ2 ($n = 8$) in CLL cells incubated with or without 1 μM CC-292 and stimulated with αIgM is shown relative to untreated stimulated cells (100%). Representative histograms of one case (CLL24) are shown. (*b*) CD69 expression on CLL cells ($n = 5$) cultured alone or co cultured for 24 h with HS-5 and HK cell lines in the presence or absence of 1 μM CC-292. (*c*) CC-292 (1 μM)-pretreated or untreated CLL cells ($n = 12$) were stimulated with αIgM and incubated for 3 h to allow migration toward CXCL12 through VCAM-1-coated transwell inserts. Migration is represented as the ratio of migrating cells and total viable cells, relative to the untreated stimulated control. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. Results are depicted as mean ± s.e.m.

in vitro (Supporting Information Table S5). In coculture systems, both HS-5 and HK cell lines protected CLL cells from spontaneous and drug-induced apoptosis ($p < 0.001$). Only the combination of CC-292 and bendamustine was found to completely abrogate this stroma-mediated protection (Fig. 3*a* and *b*). In HS-5 cocultures, mean cytotoxicity induced by CC-292 and by bendamustine was 7.7% and 31.6%, respectively, whereas that of the combination was 43.1% (Fig. 3*a*). Similarly, in HK cocultures, the cytotoxic effect of CC-292 and bendamustine was 12.1% and 26.8%, respectively, whereas that of the combination increased up to 46.5% (Fig. 3*b*). Similar results were obtained with ibrutinib in combination with bendamustine (Supporting Information Fig. S4*a* and S4*b*).

CC-292/bendamustine treatment effectively controls CLL development *in vivo*

In order to assess the *in vivo* activity of CC-292 and to validate the *in vitro* results obtained from its combination with bendamustine, we used the TCL1 AT mouse model of CLL. Leukemic TCL1 AT splenocytes were transplanted into syngeneic immunocompetent C57BL/6 N mice. After 14 days, mice presented mean TL of 50% (Supporting Information Fig. S5*a*). They were then randomized in four groups (Supporting Information Fig. S5*b*) and treated for 11 days (Fig. 4*a*). During the treatment, absolute lymphocyte counts (ALC) in blood were monitored weekly. In all treatment conditions, a decrease was detected, being this more remarkable in the combination

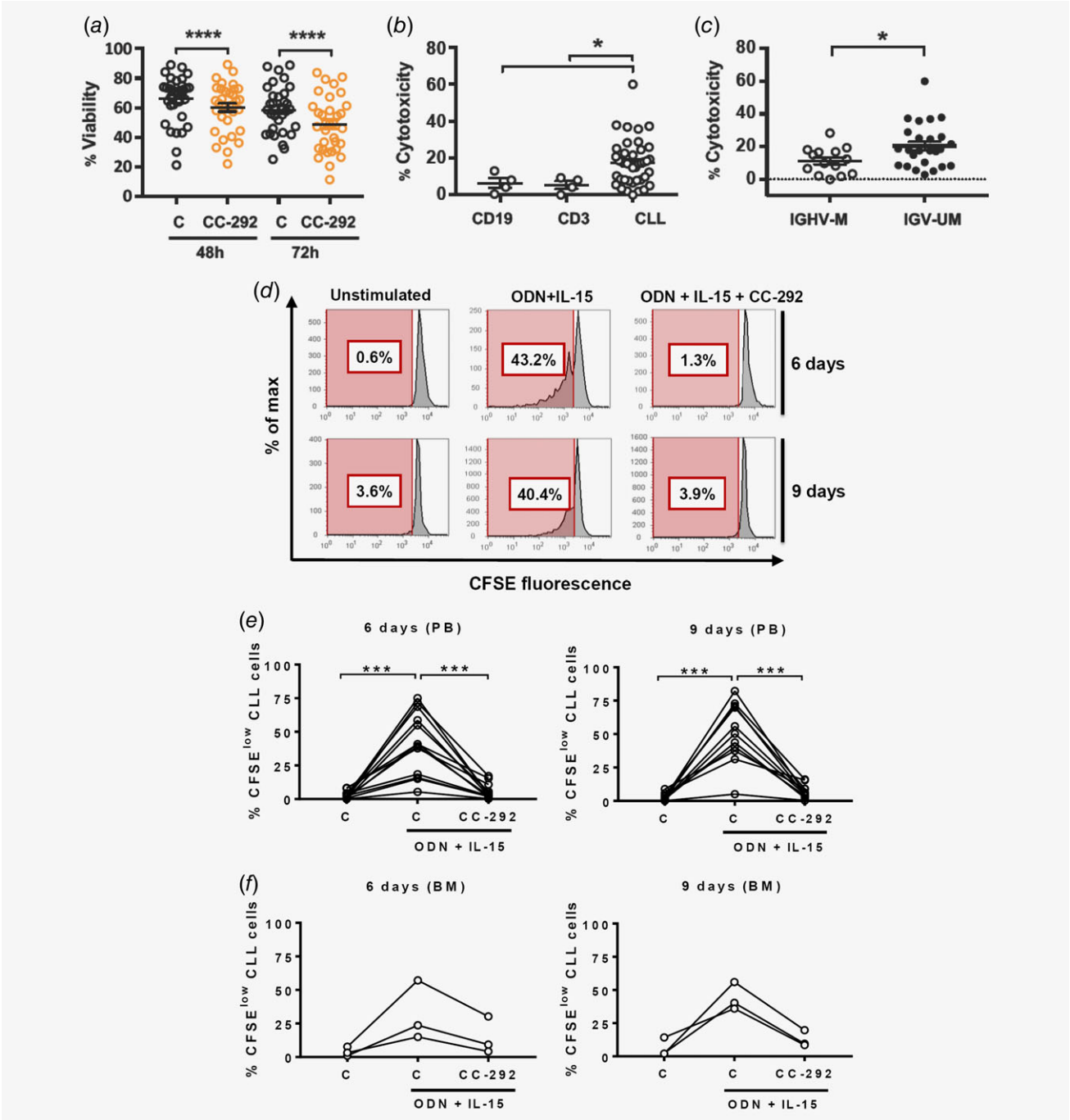

Figure 2. CC-292 exerts selective apoptosis and potently blocks proliferation in CLL cells. (*a*) CLL cell viability after 48 h and 72 h of incubation with 1 μM CC-292 quantified by Annexin-V/PI staining (n = 41). (*b*) Cytotoxicity after 72 h of incubation with 1 μM CC-292 in CLL cells (n = 41) and in $CD19^+$ and $CD3^+$ cells from healthy donors (n = 4), relative to untreated control. (*c*) Cytotoxicity after 72 h of incubation with 1 μM CC-292 in IGHV-UM CLL (n = 25) and IGHV-M CLL (n = 14) cases, relative to untreated control. (*d*) Histograms of CFSE staining in a representative CLL case (CLL15) after 6 and 9 days of incubation. (*e*) Percentage of divided $CD19^+$ viable cells in PB samples, showing a decrease in CFSE staining indicative of new cell generations, after 6 days (n = 13) and 9 days (n = 11) of incubation in unstimulated and untreated cells (C: control), and cells stimulated with ODN + IL-15, untreated or treated with 1 μM CC-292. (*f*) Percentage of divided $CD19^+$ viable cells in BM samples (n = 3) showing a decrease in CFSE staining after 6 days and 9 days of culture in the conditions previously detailed. Values are shown as dots, connected with a line in each case. $*p < 0.05$; $***p < 0.001$; $****p < 0.0001$. [Color figure can be viewed at wileyonlinelibrary.com]

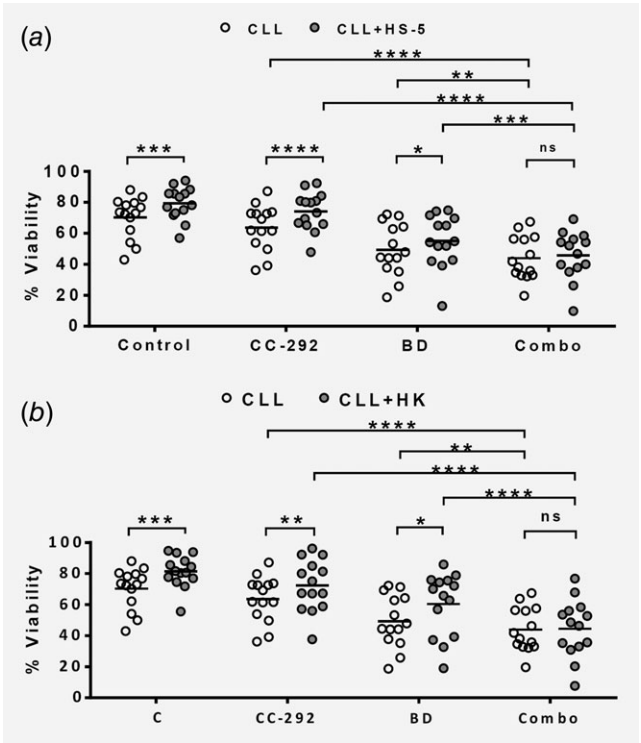

Figure 3. CC-292 and bendamustine cooperate to overcome stromal protection of CLL cells. Viability of CLL cells alone (white boxes) or cocultured (dark boxes) with (*a*) HS-5 or (*b*) HK cells after 48 h treatment with 1 μM CC-292, 25 μM bendamustine, and the drug combination, analyzed by Annexin-V/PI staining ($n = 14$). Mean ± s.e.m. is displayed. ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; *ns*, $p > 0.05$.BD: bendamustine, Combo: CC-292 + bendamustine.

treatment (Supporting Information Fig. S6*a*). Furthermore, untreated mice exhibited low red blood cell and platelet counts, which were improved after treatment with CC-292, bendamustine, or the combination (Supporting Information Fig. S6*b* and S6*c*). Untreated mice showed severe splenomegaly and hepatomegaly (Fig. 4*b*), which were significantly less severe both in CC-292 and bendamustine-treated animals (spleen weight 2.2-fold lower in CC-292 cohort ($p < 0.0001$) and 2.5-fold lower in bendamustine cohort ($p < 0.0001$), liver weight 1.6-fold lower both in CC-292 ($p < 0.0001$) and bendamustine cohorts ($p = 0.0002$)). Mice treated with the combination showed a markedly lower spleen weight of up to 5-fold less ($p < 0.0001$) and a 1.7-fold lower liver weight ($p < 0.0001$) compared to untreated animals.

We further analyzed the effect of the treatment in the lymphoid compartments (Fig. 4*c*). Bendamustine significantly reduced TL (percentage of $CD5^+$ $CD19^+$ cells out of $CD45^+$ population) in PB (1.2-fold; $p = 0.0006$) and BM compartments (2.4-fold; $p < 0.0001$). In LN, there was a tendency of fewer tumor cells in mice treated with CC-292 (1.5-fold decrease; $p = 0.057$) and the drug combination (1.4-fold; $p = 0.070$) compared to control mice.

To corroborate the antiproliferative effect of CC-292 observed *in vitro,* we quantified the percentage of Ki-67-expressing cells in the LN and BM of TCL1 AT mice (Fig. 4*d*). In the LN, each single-agent significantly decreased the percentage of Ki-67^+ leukemic cells (CC-292: 1.4-fold; $p = 0.0013$; bendamustine: 1.3-fold; $p = 0.0018$), and this effect was more pronounced with the combination of both drugs (2.5-fold; $p < 0.0001$). Similar results were obtained in the BM compartment (CC-292: 1.5-fold; bendamustine: 1.6-fold; combination: 3.6-fold; $p < 0.0001$).

CC-292 treatment alters the composition and phenotype of myeloid and T cells in the CLL microenvironment

In addition to their direct impact on tumor cells, BTK inhibitors modulate multiple cell types in the tumor microenvironment, such as tumor-associated myeloid cells and T cells.[28] Ibrutinib has impact on T-cell numbers and function due to its off-target effects on ITK, a central kinase in T-cell receptor signaling.[29,30] Furthermore, CLL development in the TCL1 model induces the accumulation of tumor-supportive monocytes with severe skewing toward $Ly6C^{low}$ patrolling monocytes.[25] Accordingly, we classified monocytes based on the expression of Ly6C, and we observed that the number of patrolling monocytes ($Ly6C^{low}$) in control mice were 2.5-fold higher than those of inflammatory monocytes ($Ly6C^{high}$) (Fig. 5*a*). Mice treated with CC-292 experienced a substantial decrease in the number of both $Ly6C^{hi}$ and $Ly6C^{low}$ monocytes ($p = 0.0016$ and $p < 0.0002$, respectively; Fig. 5*b*), with no major alteration of the patrolling-to-inflammatory ratio (Fig. 5*a*). Mice treated with bendamustine or with the combination also experienced a strong reduction in monocyte numbers, albeit the reduction was stronger in the patrolling monocyte subset ($Ly6C^{hi}$ $p = 0.0034$ and $Ly6C^{low}$ $p < 0.0001$) with bendamustine; $Ly6C^{hi}$ $p = 0.0162$ and $Ly6C^{low}$ $p < 0.0003$ with the combination; Fig. 5*b*), whereby the patrolling-to-inflammatory ratio became close to 1 (Fig. 5*a*). In addition to quantifying monocytes in blood, we further analyzed their subset distribution in spleen where the majority of these cells in leukemic mice are of patrolling phenotype.[25] In line with the data in blood, bendamustine but not CC-292 treatment reduced the percentage of patrolling monocytes in spleen, even though here the majority of monocytes remained of patrolling phenotype under all treatment conditions (Supporting Information Fig. S6*d*).

Moreover, we analyzed the effect of the treatment on T-cell populations in PB, which are known to increase along with disease course.[26,31] We detected a decrease in $CD4^+$ and $CD8^+$ T-cell numbers after either single-agent treatment ($CD8^+$: $p = 0.0128$ and $CD4^+$: $p = 0.1020$ with CC-292; $CD8^+$: $p = 0.009$ and $CD4^+$: $p = 0.0086$ with bendamustine), as well as with the drug combination ($CD8^+$: $p = 0.0034$ and $CD4^+$: $p = 0.0035$; Fig. 5*c*). While CLL development is associated with a drop in the $CD4^+/CD8^+$ cell ratio due to $CD8^+$ T-cell expansion,[30] CC-292 treatment resulted in a significant

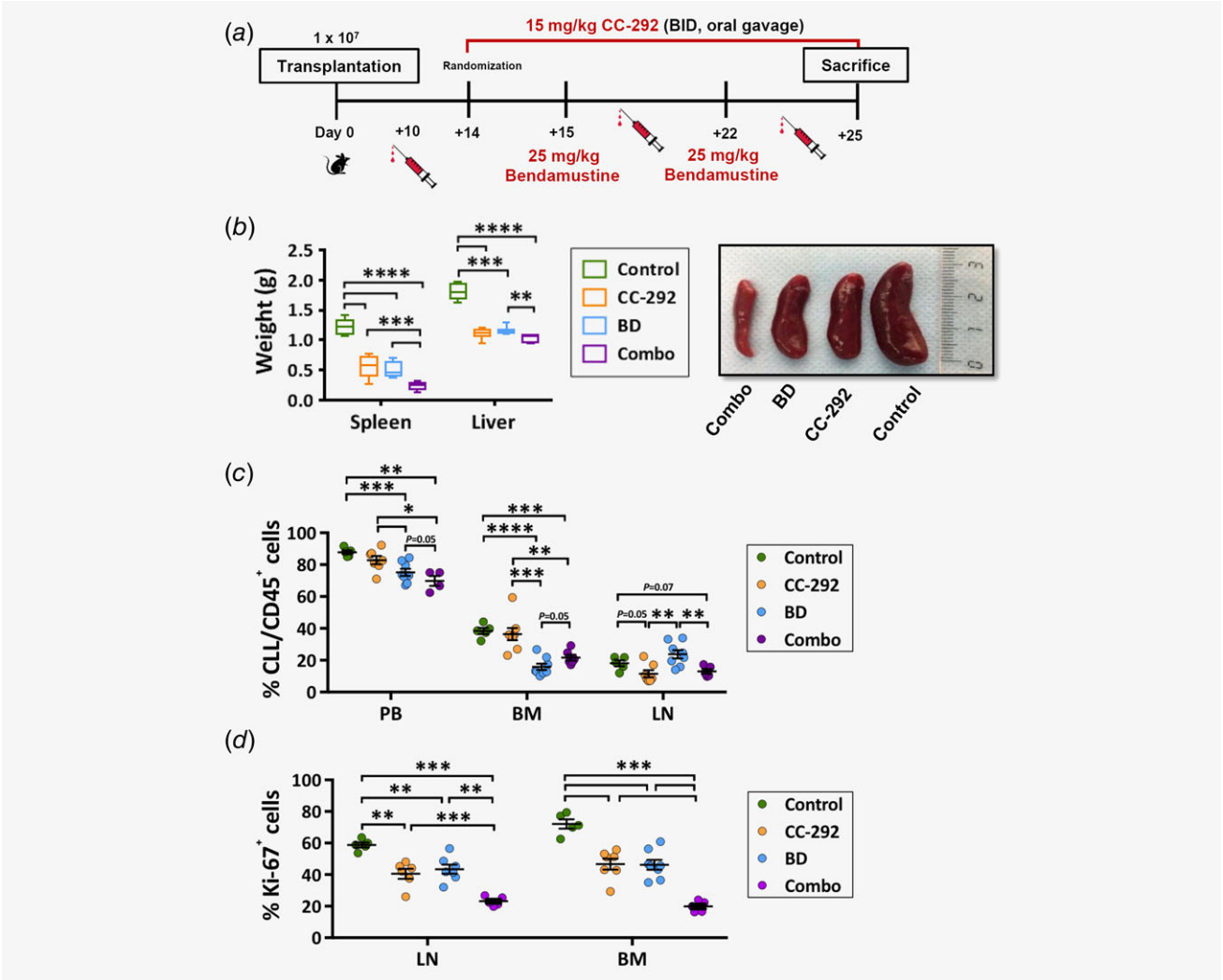

Figure 4. Combination therapy of CC-292 and bendamustine controls CLL development in the TCL1 AT model. (*a*) *In vivo* treatment schedule. (*b*) Spleen and liver weight in control and treated mice (n = 5–8). Representative examples of spleens are shown. (*c*) Tumor load (CD19$^+$/CD5$^+$ cells out of CD45$^+$ cells) in PB, BM and LN compartments (n = 4–8). (*d*) Ki-67 staining in the LN and BM compartments (n = 4–8). Results are depicted as mean ± s.e.m. ****p ‹ 0.0001; ***p ‹ 0.001; **p ‹ 0.01; *p ‹ 0.05; *ns*, p ≥ 0.05. BM: bone marrow, LN: lymph node, PB: peripheral blood.

increase in the CD4$^+$/CD8$^+$ cell ratio in PB (p = 0.0079; Supporting Information Fig. S6*e*). Collectively, these data show that CC-292 treatment results in a normalization of CLL-induced changes in monocyte and T-cell numbers.

CC-292 treatment does not affect T-cell function

We then analyzed whether the described decrease in T-cell numbers by CC-292 may negatively affect antitumor immune functions. CLL development has been shown to impact on the differentiation and function of CD4$^+$ and CD8$^+$ T-cells.[26] In line with previous reports,[26] splenic CD4$^+$ T-cells in untreated mice showed a significant expansion of the CD44^{hi} CD62L^{low} effector/memory population (Fig. 6*a*). Similarly, CD8$^+$ T-cells in untreated mice were primarily composed of antigen-experienced effector (CD127^{low} CD44$^+$) and memory (CD127^{hi} CD44^{hi}) subsets, with a drop in the percentage of the naïve (CD127^{hi} CD44$^-$) population (Fig. 6*b*). CC-292 treatment led to a mild but significant decrease in effector/memory CD4$^+$ T-cells (p = 0.0395; Fig. 6*a*) and memory CD8$^+$ T-cells (p = 0.0147; Fig. 6*b*), accompanied by an increase in naïve CD8$^+$ T-cells (p = 0.0241). Importantly, we did not detect a significant decrease in the percentage of effector CD8$^+$ T-cells (Fig. 6*b*), which have been recently described to be specifically enriched with oligoclonal T-cells which possess anti-tumor functions.[32] We further analyzed whether CC-292 impacts on the functional capacity of T-cells. Besides a slight reduction in the percentage of IFNγ-producing CD4$^+$ CD44$^+$ T-cells, CC-292-treated mice showed no significant differences in the production of effector cytokines, such as IFNγ, TNFα, or IL-2 by neither antigen-experienced CD4$^+$

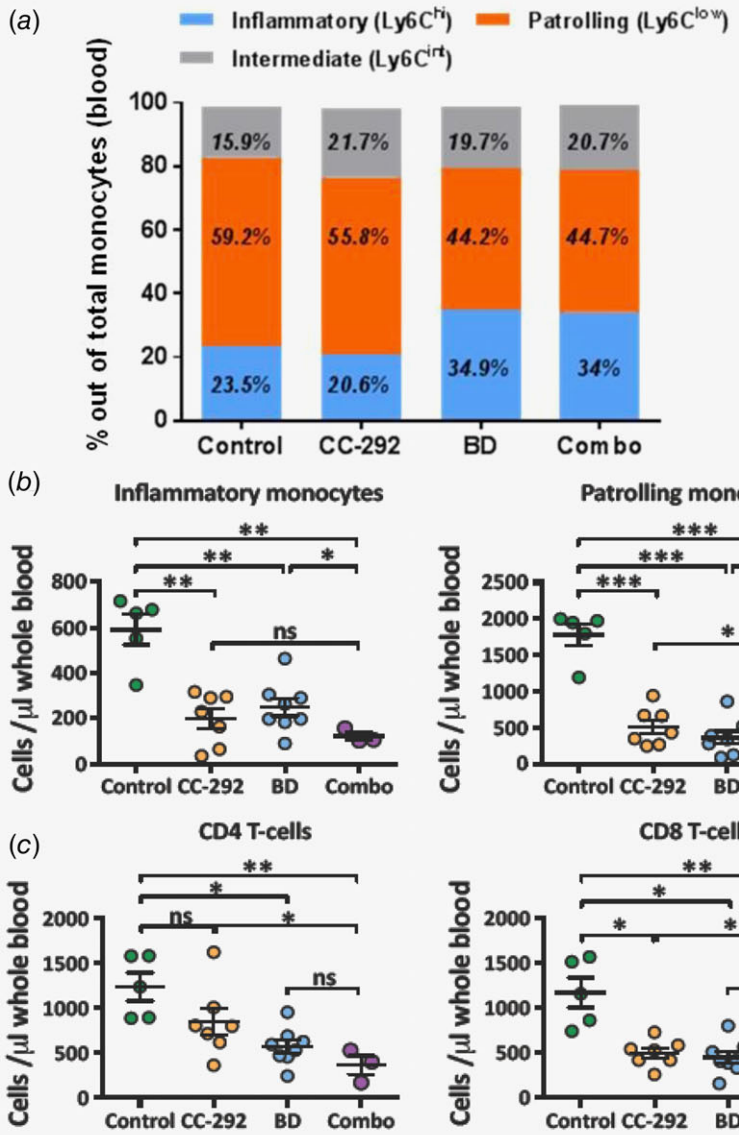

Figure 5. CC-292 normalizes the number and composition of tumor-associated myeloid and T-cells. (*a*) Percentages of inflammatory (Ly6C^{high}), patrolling (Ly6C^{low}), and intermediate monocytes (Ly6C^{int}) out of total monocytes in PB of untreated and treated mice (n = 3–8). (*b*) Presence of inflammatory (Ly6C^{high}) and patrolling (Ly6C^{low}) subsets out of total monocytes in PB of control and treated mice (n = 3–8). (*c*) Presence of CD4$^+$ and CD8$^+$ populations out of total T-cells in PB of control and treated mice (n = 3–8). Results are depicted as mean ± s.e.m. ****p ‹ 0.0001; ***p ‹ 0.001; *p ‹ 0.05; *ns*, p ≥ 0.05. BD: bendamustine, Combo: CC-292 + bendamustine.

CD44$^+$ T-cells nor effector CD8$^+$ T-cells, upon *ex vivo* stimulation (Fig. 6*c* and *d*). In addition, the *ex vivo* degranulation capacity of effector CD8$^+$ T-cells, as measured by CD107a presentation on cell surface, was even higher in CC-292-treated mice compared to control mice (p = 0.0497; Fig. 6*e*). Collectively, these data indicate that CC-292 treatment is associated with decreased T-cell numbers, albeit with no major impact on T-cell functional capacity.

Discussion

In our study, we show that spebrutinib, a highly selective small molecule inhibitor that shows greater selectivity than ibrutinib for BTK,[20] is capable of interfering with BCR signaling in CLL cells. CC-292 was able to inhibit BTK autophosphorylation and consequent PLCγ2 phosphorylation upon BCR activation in primary CLL cells, similarly as reported for ibrutinib.[33] Furthermore, as BTK plays a role in the modulation of chemokine receptors and adhesion molecules involved in B-cell homing, CC-292 completely impaired the ability of αIgM-stimulated CLL cells to attach to VCAM-1 and migrate toward CXCL12, the main chemokine involved in CLL cell homing and retention in the LN and BM microenvironments.[34] This comes in line with the rapid reduction of lymphadenopathy with accompanying peripheral lymphocytosis observed during an early clinical trial of

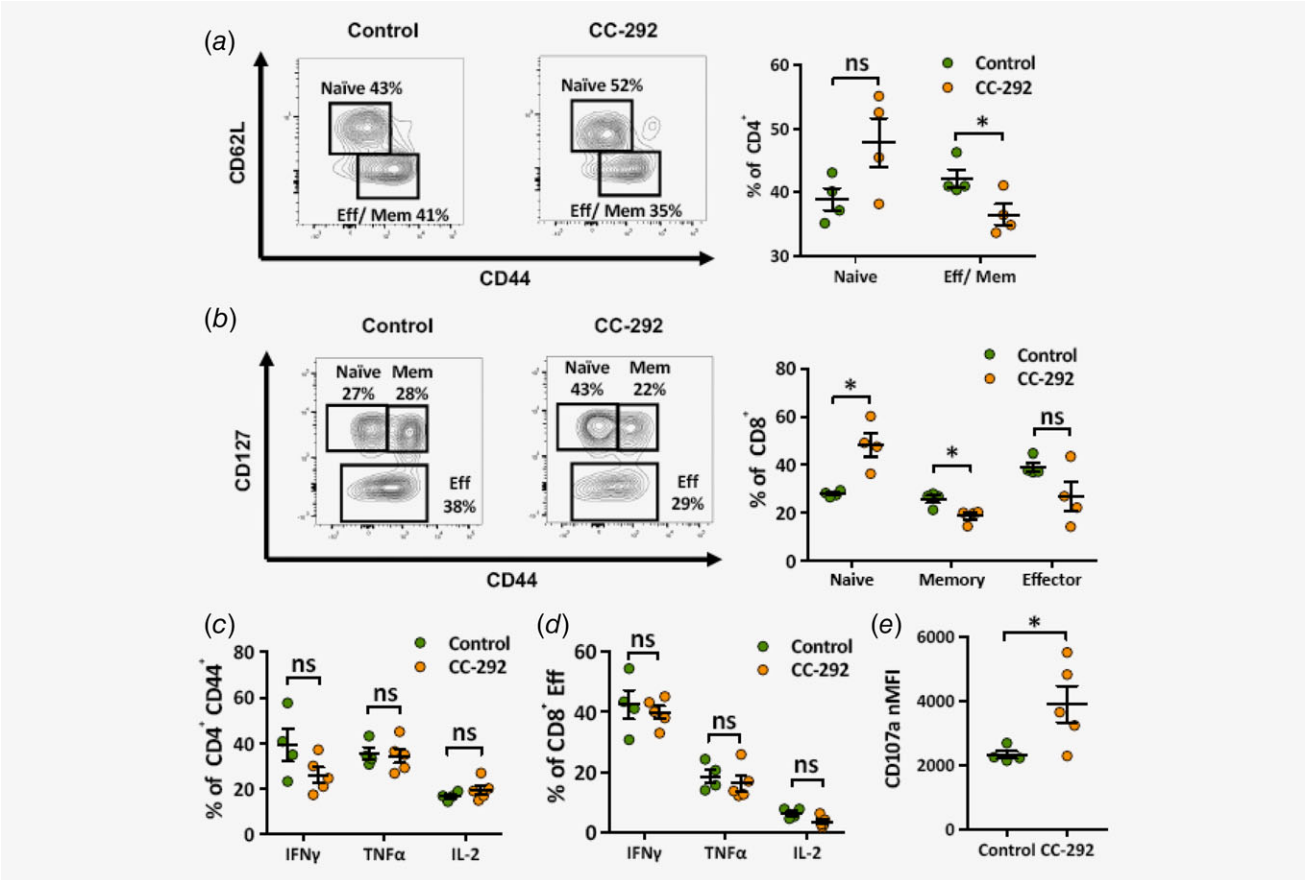

Figure 6. CC-292 repairs T-cell skewing toward antigen experienced T cells in the CLL microenvironment with no impact on T-cell functional capacity. (*a*) Representative flow cytometry plots and percentage of naïve ($CD44^{-}$ $CD62L^{hi}$) and effector/memory ($CD44^{hi}$ $CD62L^{low}$) spleen $CD4^{+}$ T-cell populations in control and CC-292-treated TCL1 AT mice ($n = 4$). (*b*) Representative flow cytometry plots and percentage of effector ($CD127^{low}$ $CD44^{+}$), memory ($CD127^{hi}$ $CD44^{hi}$), and naïve ($CD127^{hi}$ $CD44^{-}$) spleen $CD8^{+}$ T-cells in control and CC-292-treated mice ($n = 4$). (*c*) Cytokine production in antigen-experienced $CD44^{+}/CD4^{+}$ cells upon *ex vivo* stimulation with PMA/Ionomycin. (*d*) Cytokine production and (*e*) degranulation capability measured by normalized mean fluorescence intensity (nMFI) of CD107a in $CD127^{low}/CD44^{+}$ effector $CD8^{+}$T-cells ($n = 4–5$). *$p < 0.05$; *ns*, $p \geq 0.05$. [Color figure can be viewed at wileyonlinelibrary.com]

spebrutinib in CLL patients.[21] We demonstrated that CC-292 is able to completely inhibit proliferation in CLL cells. In contrast, the *in vitro* cytotoxic effect of CC-292, albeit significant, was modest and lower than that of ibrutinib. Similarly, a cytostatic effect of CC-292 with a marginal pro-apoptotic effect was previously reported in myeloma cells[35] and mantle cell lymphoma cells.[36] Altogether, our observations suggest that similarly to the effect of ibrutinib,[33] inhibition of cell proliferation, rather than apoptosis induction, is one of the primary mechanisms of CC-292 antitumor effect against CLL leukemic cells.

Given that a phase I clinical study of single-agent spebrutinib in relapsed or refractory CLL patients has shown limited activity[21] compared to ibrutinib[15] or acalabrutinib[37] that may be due to its superior specificity for BTK and lower off-target effect toward ITK,[20] we investigated whether a greater therapeutic effect could be achieved in combination with other drugs. In a previous study, we identified the expression of the early activation marker CD69 as an independent predictor of resistance to bendamustine and we described that ibrutinib is able to down-regulate CD69 levels and to sensitize CLL cells to bendamustine.[11] Bendamustine is a cytotoxic drug sharing structural similarities to alkylating agents and purine analogues. The unique structural and mechanistic features of bendamustine differentiate it from other alkylating agents, providing increased stability and potency in DNA cross linking and subsequent cytotoxicity resulting in cell death. Furthermore, it has been shown to be active against both active and quiescent cells.[38] Recently, a phase III trial showed that the combination of BR with ibrutinib improved the outcome of CLL patients compared to BR alone, with a faster and deeper remission with more complete responses.[39] The ability of bendamustine to quickly reduce TL, suggests a role as a debulking agent in the era of targeted therapies.[40,41] In contrast to that, CC-292 blocks BTK-mediated signaling, including BCR, TLR and chemokine receptor mediated signals, all of which are known to be of relevance for CLL cell survival, proliferation, migration and homing. CC-292, similarly to ibrutinib is able to decrease CD69 expression and to enhance

the cytotoxic effect of bendamustine. In common with BCR-directed drugs, we observed that CC-292 disrupts CLL chemotaxis. This might lead to the egress of CLL cells from lymphoid tissues into the blood stream that has been described in clinical studies. In the blood stream, signals for cell activation are weaker and bendamustine might therefore exert higher cytotoxic activity. Therefore, this strategy might be useful in patients with a higher tumor load and the necessity to achieve a quick response.[12]

To confirm our *in vitro* results, we analyzed the efficacy of the combination *in vivo*, using the TCL1 AT mouse model of CLL that recapitulates tumor-microenvironment interactions. Mice treated with CC-292 experienced no detectable increase in lymphocytosis, similarly to the results observed in TCL-1 mice treated with the BTK inhibitor acalabrutinib.[42] This suggests that most tumor cell death might occur within the tissues. Accordingly, CC-292 was more active in the LN compartment, whereas bendamustine was more effective in the PB and BM compartments. Mice treated with the drug combination showed a dramatic reduction in the infiltration of all affected lymphoid compartments (PB, LN, BM and spleen). This went hand in hand with a decrease in proliferation that confirmed our *in vitro* observations in primary CLL cells.

In addition to a direct impact on tumor cells, genetic or pharmacological inhibition of BTK can modulate multiple cell types in the tumor microenvironment.[43,44] In this way, BTK inhibition induces reprogrammed tumor-associated myeloid cells toward antitumor phenotypes.[28] Recently, Hanna *et al.* reported that CLL development in the TCL1 AT model induces the accumulation of tumor-supportive monocytes with severe skewing toward patrolling monocytes,[25] and they showed that myeloid cell depletion can result in a repair of innate immune cell phenotypes and a partial resolution of systemic inflammation. In our study, CC-292 treatment led to a substantial decrease in the number of both inflammatory and patrolling monocytes, and its combination with bendamustine led to a rebalance in the ratio of patrolling-to-inflammatory monocytes, suggesting some immunomodulatory effects of CC-292 on CLL-associated myeloid cells.

Furthermore, CC-292 alone or in combination, normalized the numbers of T-cells, while maintaining their functional capacities. In contrast to ibrutinib, CC-292 has lower off-target effects on multiple kinases including ITK, whose inhibition can modulate multiple immune cell types. In natural killer cells, ITK inhibition by ibrutinib impairs Fc receptor-mediated cell functions and antagonizes rituximab cytotoxicity.[45] Furthermore, ibrutinib inhibition of ITK in T-cells abrogates TCR signaling, and induces $CD4^+$ T-cells polarization toward Th1 phenotypes.[29] In contrast, we have only observed a slight, but not a significant change in the ability of $CD4^+$ T-cells to produce the Th1 master cytokine IFNγ in CC-292-treated mice. Moreover, while decreased $CD8^+$ T-cell activation has been observed in ibrutinib-treated patients,[46] no significant changes in the number or function of anti-tumor effector $CD8^+$ T-cells were observed in CC-292-treated mice, indicating that CC-292 seems to preserve the functional capacity of T-cells.

In summary, our data suggest that CC-292 is effective in disrupting BCR signaling and inhibiting tumor cell activation, proliferation, and chemotaxis, similarly to what was previously shown for ibrutinib. In combination with bendamustine, CC-292 is able to overcome microenvironment-mediated chemoresistance, normalize immune cell composition, and significantly reduce the tumor burden of mice adoptively transferred with TCL1 leukemic cells. Our results identify CC-292 as a potent inhibitor of BTK with promising activity in combination with bendamustine in CLL. As BTK resistance or intolerance to ibrutinib is a clinical challenge, further studies combining alternative BTK inhibitors with bendamustine are warranted.

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Author's contributions

DC, MS, ELG, BSH, designed the experiment; ELV, BSH, HY, VR, MLR, AG, AVC, NV, PPG, LR, developed methodology; MA. NV, JD, MLG, organized the clinical materials;

ELV, BSH, HY, Analysis and interpretation of data; ELV, BSH, MS, DC, wrote the paper. VA, PPG, JD, NV, LR, PL, MLG and EC, review the study. DC and MS supervised the study.

References

1. Burger JA, Chiorazzi N. B cell receptor signaling in chronic lymphocytic leukemia. *Trends Immunol* 2013;34:592–601.
2. Maas A, Hendriks RW. Role of Bruton's tyrosine kinase in B cell development. *Dev Immunol* 2001; 8:171–81.
3. Mohamed AJ, Yu L, Backesjo CM, et al. Bruton's tyrosine kinase (Btk): function, regulation, and transformation with special emphasis on the PH domain. *Immunol Rev* 2009;228:58–73.
4. Jain N, O'Brien S. Initial treatment of CLL: integrating biology and functional status. *Blood* 2015; 126:463–70.
5. Eichhorst B, Fink AM, Bahlo J, et al. First-line chemoimmunotherapy with bendamustine and rituximab versus fludarabine, cyclophosphamide, and rituximab in patients with advanced chronic lymphocytic leukaemia (CLL10): an international, open-label, randomised, phase 3, non-inferiority trial. *Lancet Oncol* 2016;17: 928–42.
6. Cheson BD, Leoni L. Bendamustine: mechanism of action and clinical data. *Clin Adv Hematol Oncol* 2011;9:1–11.

7. Cheson BD. Bendamustine in CLL: how quickly will today's consensus become tomorrow's memory? *Leuk Res* 2014;38:1267–8.
8. Roue G, Lopez-Guerra M, Milpied P, et al. Bendamustine is effective in p53-deficient B-cell neoplasms and requires oxidative stress and caspase-independent signaling. *Clin Cancer Res* 2008;14:6907–15.
9. Fischer K, Cramer P, Busch R, et al. Bendamustine combined with rituximab in patients with relapsed and/or refractory chronic lymphocytic leukemia: a multicenter phase II trial of the German chronic lymphocytic leukemia study group. *J Clin Oncol* 2011;29:3559–66.
10. Fischer K, Cramer P, Busch R, et al. Bendamustine in combination with rituximab for previously untreated patients with chronic lymphocytic leukemia: a multicenter phase II trial of the German chronic lymphocytic leukemia study group. *J Clin Oncol* 2012;30:3209–16.
11. Montraveta A, Lee-Verges E, Roldan J, et al. CD69 expression potentially predicts response to bendamustine and its modulation by ibrutinib or idelalisib enhances cytotoxic effect in chronic lymphocytic leukemia. *Oncotarget* 2016;7:5507–20.
12. Al-Sawaf O, Cramer P, Goede V, et al. Bendamustine and its role in the treatment of unfit patients with chronic lymphocytic leukaemia: a perspective review. *Ther Adv Hematol* 2017;8:197–205.
13. Burger JA, Tedeschi A, Barr PM, et al. Ibrutinib as initial therapy for patients with chronic lymphocytic leukemia. *N Engl J Med* 2015;373:2425–37.
14. Brown JR, Hillmen P, O'Brien S, et al. Extended follow-up and impact of high-risk prognostic factors from the phase 3 RESONATE study in patients with previously treated CLL/SLL. *Leukemia* 2018;32:83–91.
15. Byrd JC, Furman RR, Coutre SE, et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N Engl J Med* 2013;369:32–42.
16. O'Brien S, Jones JA, Coutre SE, et al. Ibrutinib for patients with relapsed or refractory chronic lymphocytic leukaemia with 17p deletion (RESONATE-17): a phase 2, open-label, multicentre study. *Lancet Oncol* 2016;17:1409–18.
17. Woyach JA, Furman RR, Liu TM, et al. Resistance mechanisms for the Bruton's tyrosine kinase inhibitor ibrutinib. *N Engl J Med* 2014;370:2286–94.
18. Jain P, Keating M, Wierda W, et al. Outcomes of patients with chronic lymphocytic leukemia after discontinuing ibrutinib. *Blood* 2015;125:2062–7.
19. Burger JA, Buggy JJ. Bruton tyrosine kinase inhibitor ibrutinib (PCI-32765). *Leuk Lymphoma* 2013;54:2385–91.
20. Evans EK, Tester R, Aslanian S, et al. Inhibition of Btk with CC-292 provides early pharmacodynamic assessment of activity in mice and humans. *J Pharmacol Exp Ther* 2013;346:219–28.
21. Brown JR, Harb WA, Hill BT, et al. Phase I study of single-agent CC-292, a highly selective Bruton's tyrosine kinase inhibitor, in relapsed/refractory chronic lymphocytic leukemia. *Haematologica* 2016;101:e295–e8.
22. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood* 2016;127:2375–90.
23. Mongini PK, Gupta R, Boyle E, et al. TLR-9 and IL-15 synergy promotes the in vitro clonal expansion of chronic lymphocytic leukemia B cells. *J Immunol* 2015;195:901–23.
24. Bichi R, Shinton SA, Martin ES, et al. Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression. *Proc Natl Acad Sci U S A* 2002;99:6955–60.
25. Hanna BS, McClanahan F, Yazdanparast H, et al. Depletion of CLL-associated patrolling monocytes and macrophages controls disease development and repairs immune dysfunction in vivo. *Leukemia* 2016;30:570–9.
26. McClanahan F, Hanna B, Miller S, et al. PD-L1 checkpoint blockade prevents immune dysfunction and leukemia development in a mouse model of chronic lymphocytic leukemia. *Blood* 2015;126:203–11.
27. Betts MR, Brenchley JM, Price DA, et al. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J Immunol Methods* 2003;281:65–78.
28. Gunderson AJ, Kaneda MM, Tsujikawa T, et al. Bruton tyrosine kinase-dependent immune cell cross-talk drives pancreas cancer. *Cancer Discov* 2016;6:270–85.
29. Dubovsky JA, Beckwith KA, Natarajan G, et al. Ibrutinib is an irreversible molecular inhibitor of ITK driving a Th1-selective pressure in T lymphocytes. *Blood* 2013;122:2539–49.
30. Long M, Beckwith K, Do P, et al. Ibrutinib treatment improves T cell number and function in CLL patients. *J Clin Invest* 2017;127:3052–64.
31. Hofbauer JP, Heyder C, Denk U, et al. Development of CLL in the TCL1 transgenic mouse model is associated with severe skewing of the T-cell compartment homologous to human CLL. *Leukemia* 2011;25:1452–8.
32. Hanna BS, Roessner PM, Yazdanparast H, et al. Control of chronic lymphocytic leukemia development by clonally expanded CD8+ T-cells that undergo functional exhaustion in secondary lymphoid tissues. *Leukemia* 2018 Sep 28. https://doi.org/10.1038/s41375-018-0250-6. [Epub ahead of print]
33. Herman SE, Gordon AL, Hertlein E, et al. Bruton tyrosine kinase represents a promising therapeutic target for treatment of chronic lymphocytic leukemia and is effectively targeted by PCI-32765. *Blood* 2011;117:6287–96.
34. Burger JA, Burger M, Kipps TJ. Chronic lymphocytic leukemia B cells express functional CXCR4 chemokine receptors that mediate spontaneous migration beneath bone marrow stromal cells. *Blood* 1999;94:3658–67.
35. Eda H, Santo L, Cirstea DD, et al. A novel Bruton's tyrosine kinase inhibitor CC-292 in combination with the proteasome inhibitor carfilzomib impacts the bone microenvironment in a multiple myeloma model with resultant antimyeloma activity. *Leukemia* 2014;28:1892–901.
36. Vidal-Crespo A, Rodriguez V, Matas-Cespedes A, et al. The Bruton tyrosine kinase inhibitor CC-292 shows activity in mantle cell lymphoma and synergizes with lenalidomide and NIK inhibitors depending on nuclear factor-kappaB mutational status. *Haematologica* 2017;102:e447–e51.
37. Byrd JC, Harrington B, O'Brien S, et al. Acalabrutinib (ACP-196) in relapsed chronic lymphocytic leukemia. *N Engl J Med* 2016;374:323–32.
38. Leoni LM, Hartley JA. Mechanism of action: the unique pattern of bendamustine-induced cytotoxicity. *Semin Hematol* 2011;48(Suppl 1):S12–23.
39. Chanan-Khan A, Cramer P, Demirkan F, et al. Ibrutinib combined with bendamustine and rituximab compared with placebo, bendamustine, and rituximab for previously treated chronic lymphocytic leukaemia or small lymphocytic lymphoma (HELIOS): a randomised, double-blind, phase 3 study. *Lancet Oncol* 2016;17:200–11.
40. Gordon MJ, Lewis LD, Brown JR, et al. Bendamustine hydrochloride in patients with B-cell malignancies who have comorbidities - is there an optimal dose? *Expert Rev Hematol* 2017;10:707–18.
41. Cheson BD. What is the role of chemotherapy in patients with chronic lymphocytic leukemia? *Clin Lymphoma Myeloma Leuk* 2017;17:723–7.
42. Herman SEM, Montraveta A, Niemann CU, et al. The Bruton tyrosine kinase (BTK) inhibitor Acalabrutinib demonstrates potent on-target effects and efficacy in two mouse models of chronic lymphocytic leukemia. *Clin Cancer Res* 2017;23:2831–41.
43. Byrd JC, Furman RR, Coutre SE, et al. Three-year follow-up of treatment-naive and previously treated patients with CLL and SLL receiving single-agent ibrutinib. *Blood* 2015;125:2497–506.
44. Hanna BS, Rossner PM, Iskar M, et al. IL-10 receptor deficiency aggravates exhaustion of CD8+ T-cells and impedes their control of chronic lymphocytic leukemia. *Blood* 2017;130:385 (abstract).
45. Kohrt HE, Sagiv-Barfi I, Rafiq S, et al. Ibrutinib antagonizes rituximab-dependent NK cell-mediated cytotoxicity. *Blood* 2014;123:1957–60.
46. Niemann CU, Herman SE, Maric I, et al. Disruption of in vivo chronic lymphocytic leukemia tumor-microenvironment interactions by Ibrutinib--findings from an investigator-initiated phase II study. *Clin Cancer Res* 2016;22:1572–82.