Venetoclax does not impair activated T cell proliferation

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Allogeneic hematopoietic cell transplantation (allo-HCT) is the standard consolidation therapy for patients with intermediate or high-risk acute myeloid leukemia (AML). However, while patients' outcomes after allo-HCT have significantly improved over the last decade, relapse of the underlying malignancy remains the leading cause of morbidity and mortality. Therefore, development of safe and effective therapeutic strategies to prevent or treat AML relapse is essential[1]. Donor lymphocyte infusion (DLI), a well-established treatment of relapsed AML, seems to be an effective prophylactic treatment alone or in combination with the hypomethylating agent azacytidine[2]. Furthermore, maintenance therapy with FLT3 inhibitors seems to be effective in reducing the risk of relapse of FLT3-ITD mutated AML[3].

The orally available Bcl-2 inhibitor venetoclax, combined with a hypomethylating agent has shown efficacy in newly diagnosed and relapsed or refractory AML[4]. Furthermore, Byrne et al. recently reported the use of venetoclax combined with a hypomethylating agent or low-dose aracytine in 21 patients with relapsed AML after allo-HCT[5]. Results were promising with an overall response rate of 42%, including five complete remissions and three complete remissions with incomplete count recovery, among 19 evaluable patients. Therefore, maintenance after allo-HCT with venetoclax + azacytidine is currently being evaluated in large phase 2 and phase 3 studies (NCT04128501, NCT04161885).

Nevertheless, since Bcl-2 is important for effector and memory T cell responses[6] and venetoclax can cause reductions in T cell number[7], it raises the question of the impact of venetoclax on the T cell mediated graft-versus-leukemia (GVL) effect after allo-HCT and after DLI. Thus, the aim of this study was to evaluate the *in-vitro* effect of venetoclax on alloreactive T cells in the graft-versus-host disease (GVHD)/GVL balance after allo-HCT.

We first evaluated the effect of venetoclax on activated T cells. T cell activation was achieved by incubating peripheral blood mononuclear cells (PBMCs) with anti-CD3 and anti-CD28 antibodies (Abs) in the presence of different clinically relevant concentrations of venetoclax or vehicle control for 24 hours (online supplemental methods, Figure 1). The viability of the T cell subpopulations (CD3+CD4+, CD3+CD8+) was evaluated by flow cytometry. No difference in viability was observed when the cells were treated with venetoclax or vehicle control for either CD4+ or CD8+ T cells (Figure 1A, B). Furthermore, as a control we evaluated the effect of venetoclax on activated

B cells. PBMCs were activated by CpG-B and exposed to the same concentrations of venetoclax or vehicle control for 24 hours. A significant decrease in viability of CD19+ B cells was observed in the presence of the highest concentrations of 1 mM and 3 mM of venetoclax (Figure 1C), in agreement with previously published data on B cells[8]. Overall, our findings indicate, that in contrast to activated B cells, venetoclax does not appear to have an effect on activated T cells. Furthermore, we did not find any effect of venetoclax, even at the highest concentration used compared to vehicle control on the proliferation of T cells activated by anti CD3/CD28 evaluated by CFSE assay (Figure 1D).

In addition, we evaluated the effect of venetoclax on the induction of apoptosis in T cells. For this purpose, cells were treated after activation for 24 hours with different concentrations of venetoclax. Apoptosis was evaluated by labelling with annexin V and a viability marker. Venetoclax did not induce T cell apoptosis compared to the vehicle control, even at the highest concentrations (Figure E). As a control, we also evaluated induction of apoptosis on B cells and found that the highest concentrations of venetoclax (1 and 3 mM) significantly increased B cell apoptosis as compared to the vehicle control (Figure 1E). Given the established role of Bcl-2 in T cell homeostasis[6] and the reduced T cell number in venetoclax treated patients[7], we compared Bcl-2 expression in activated T and B cells to account for the absence of effect of the Bcl-2 inhibitor venetoclax on T cells compared to B cells. Thus, we performed Bcl-2 intracellular labeling and found that Bcl-2 expression was significantly higher in activated B cells compared to T cells, (Figure 1F), explaining the differential effect of the Bcl-2 inhibitor on activated T and B cells.

Overall, our findings suggest that use of venetoclax to prevent or treat AML relapse after allo-HCT has no detrimental effect on the T cell mediated GVL effect. Furthermore, DLI is an important strategy in the armamentarium of post allo-HCT AML management[1]. DLI consisted of the administration of a selected dose of CD3+ T cells to expand the T cell repertoire and enhance the GVL effect. Therefore, based on our results, venetoclax will not impair DLI efficacy and they can be safely combined.

Regarding acute GVHD, we do not expect venetoclax to be protective and we cannot exclude that the enhanced GVL effect will come at the cost of an increased risk of acute GVHD. Nevertheless, as already known, we find that venetoclax induce activated B cell depletion. Given the important role of B cells in chronic GVHD pathophysiology[9], this suggest that venetoclax may contribute to

chronic GVHD prevention. We acknowledge that we did not evaluate the impact of venetoclax on other important immune cell subsets involved in the GVHD/GVL balance, in particular regulatory T cells and NK cells. Further studies will be necessary, including *in-vivo* studies, to decipher the exact effect of venetoclax on the different immune subsets involved in GVHD/GVL balance.

Overall, use of venetoclax to prevent or to treat AML relapse after allo-HCT seems to be a promising therapeutic strategy that does not impair the GVL effect and may enhance the allogeneic effect. This paves the way for future clinical and immunological studies in these patients and will be important in understanding the possible immunomodulatory effect of venetoclax in the post allo-HCT setting.

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Conflicts of Interest

Mohamad Mohty reports grants and/or lecture honoraria from Janssen, Sanofi, MaaT Pharma, JAZZ Pharmaceuticals, Celgene, Amgen, BMS, Takeda, Pfizer, and Roche.The other authors did not disclose any relevant conflict of interest in relation to this work.Florent Malard reports lecture honoraria from Therakos/Mallinckrodt, Biocodex, Janssen, Keocyt, Sanofi, JAZZ Pharmaceuticals, and Astellas, all outside the submitted work.

Authorship contributions

All authors listed on the manuscript have contributed substantially to this work. LS, BG, MM and FM designed the study, LS, BG, LR, NS and YY contributed the flow cytometry data, and LS and FM performed the statistical analysis. LS, BG and FM prepared the manuscript and figures for publication. All authors

Reference

1. Dholaria B, Savani BN, Hamilton BK, Oran B, Liu HD, Tallman MS *et al.* Hematopoietic Cell Transplantation in the Treatment of Newly Diagnosed Adult Acute Myeloid Leukemia: An Evidence-Based Review from the American Society of Transplantation and Cellular Therapy. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation* 2020.

2. Guillaume T, Malard F, Magro L, Labopin M, Tabrizi R, Borel C *et al.* Prospective phase II study of prophylactic low-dose azacitidine and donor lymphocyte infusions following allogeneic hematopoietic stem cell transplantation for high-risk acute myeloid leukemia and myelodysplastic syndrome. *Bone marrow transplantation* 2019; **54:** 1815-1826.

3. Bazarbachi AH, Al Hamed R, Malard F, Mohty M, Bazarbachi A. Allogeneic transplant for FLT3-ITD mutated AML: a focus on FLT3 inhibitors before, during, and after transplant. *Ther Adv Hematol* 2019; **10:** 2040620719882666.

4. DiNardo CD, Maiti A, Rausch CR, Pemmaraju N, Naqvi K, Daver NG *et al.* 10-day decitabine with venetoclax for newly diagnosed intensive chemotherapy ineligible, and relapsed or refractory acute myeloid leukaemia: a single-centre, phase 2 trial. *The Lancet. Haematology* 2020.

5. Byrne M, Danielson N, Sengsayadeth S, Rasche A, Culos K, Gatwood K *et al.* The use of venetoclax-based salvage therapy for post-hematopoietic cell transplantation relapse of acute myeloid leukemia. *American journal of hematology* 2020; **95:** 1006-1014.

6. Wojciechowski S, Tripathi P, Bourdeau T, Acero L, Grimes HL, Katz JD *et al.* Bim/Bcl-2 balance is critical for maintaining naive and memory T cell homeostasis. *The Journal of experimental medicine* 2007; **204:** 1665-1675.

7. Lu P, Fleischmann R, Curtis C, Ignatenko S, Clarke SH, Desai M *et al.* Safety and pharmacodynamics of venetoclax (ABT-199) in a randomized single and multiple ascending dose study in women with systemic lupus erythematosus. *Lupus* 2018; **27:** 290-302.

8. Khaw SL, Mérino D, Anderson MA, Glaser SP, Bouillet P, Roberts AW *et al.* Both leukaemic and normal peripheral B lymphoid cells are highly sensitive to the selective pharmacological inhibition of prosurvival Bcl-2 with ABT-199. *Leukemia* 2014; **28:** 1207-1215.

9. Malard F, Labopin M, Yakoub-Agha I, Chantepie S, Guillaume T, Blaise D *et al.* Rituximab-based first-line treatment of cGVHD after allogeneic SCT: results of a phase 2 study. *Blood* 2017; **130:** 2186-2195.

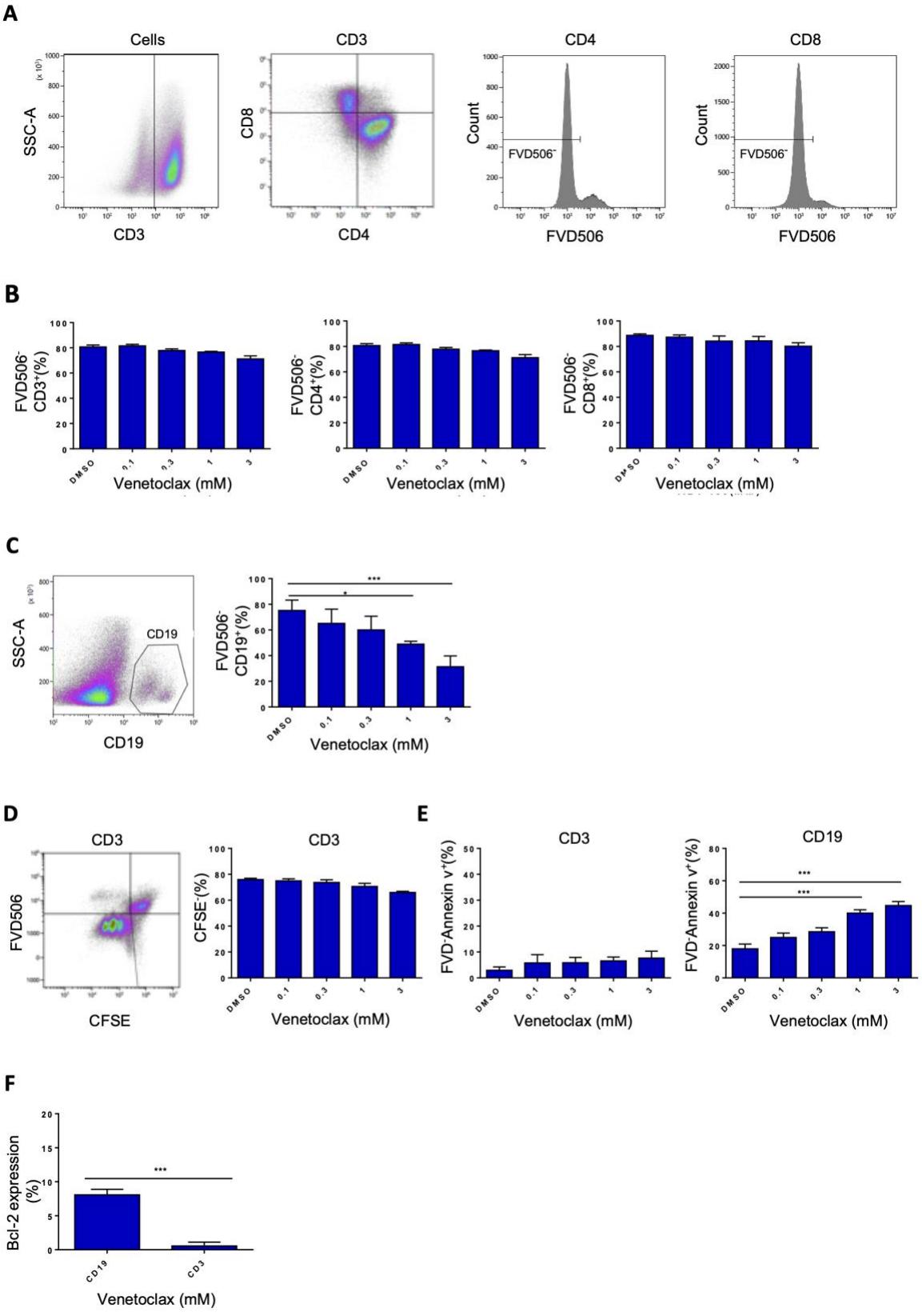
Figure legend

Figure 1: Effect of venetoclax on T cells and B cells

(A): Representative example of gating strategy for viability marker FVD506 analysis on CD4+ and CD8+ T cells. (B) The graphs represent the mean +/- SD of the % $FVD506^-$ cells among the CD3 (left panel), CD4 (middle panel) or CD8 T cells (right panel) treated with the indicated doses of venetoclax for 3 independent experiments.

(C) The graphs represent the mean +/- SD of the % $FVD506^-$ cells among $CD19^+$ B cells after treatment with the indicated doses of venetoclax for 3 independent experiments, the % of viable $FVD506^-$ cells was obtained in the $CD19^+$ B cells gate (left panel). **(D):** Effect of venetoclax on the proliferation of activated T cells. A cytometry plot showing CFSE dilution and FVD506 expression on CD3 T cells. The graph represents the mean +/- SD for 3 independent experiments of the % $FVD506^-$ $CFSE^-$ cells among the $CD3^+$ T cells treated with the indicated doses of venetoclax (right panel). **(E): Effect of venetoclax on apoptosis of T and B cells.** The graphs represents the mean +/- SD for 3 independent experiments of the % apoptotic cells FVD-Annexin V+ cells among $CD3^+$ T cells (left panel) and $CD19^+$ B cells (right panel) after treatment with the indicated doses of venetoclax.**(F):** The graph represents the Bcl-2 expression in activated T and B cells (* $p \leq 0.05$,*** $p \leq 0.001$).

Figure 1.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Supplemental data

Material and methods

Venetoclax Preparation

The different concentrations of venetoclax (ABT-199) (Euromedex) were prepared between 0.8 and 3 mM. The therapeutic concentration ranged from 0.05 mM to 2 mM[1]. Venetoclax was dissolved in DMSO (10mM) which had a concentration of 0.1% in all experiments.

Activation of T cells in vitro

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors from "Etablissement Français du Sang", Paris Saint-Antoine-Crozatier. PBMCs were isolated with a standard gradient centrifugation procedure on a lymphocyte separation medium (Lymphosep separation media, Dutscher, Issy-les-Moulineaux, France).

PBMCs were cultured at 500,000 cells/well in 96-well round bottom microplate) in RPMI medium (Eurobio) supplemented with 10% Fetal Bovine Serum (FBS),2mM L-glutamine, 100 U/mL penicillin, 100 µg/ml streptomycin, 1mM sodium pyruvate (Eurobio) and non-essential amino acids (MEM, Life Technologies). The wells were previously coated with anti-CD3 at 10 µg/ml (LEAF Purified anti-human CD3, clone OKT3, Biolegend). The PBMCs were activated in the presence of anti-CD28 (5µg/mL, Anti-Human CD28 Functional Grade Purified, clone CD28.2, eBioscience). After 3 days of culture, the cells were treated with the indicated concentrations of venetoclax ranging from 0.1 to 3 mM for 24 h and then analyzed.

Activation of B cells in vitro

PBMCs from healthy donors were cultured at 500,000 cells/well (96-well round bottom microplate, Sarstedt) in RPMI medium supplemented with 10% FBS, 2mM L-glutamine, 100 U/mL penicillin, 100 µg/ml streptomycin, 1mM sodium pyruvate (Eurobio) and non-essential amino acids (MEM, Life Technologies). PBMCs were activated in the presence of 1µg/mL of CpG-B ODN 2006 (Miltenyi Biotec, Paris, France) for 24 hours, and treated with different doses of ventoclax and then analyzed by flow cytometry.

Proliferation analysis

PBMCs were incubated for 10 min with 1µM of CFSE (Invitrogen) in a light-free environment at room temperature. To stop the reaction, complete cold medium is added and the tube is placed on ice. Then the cells are washed three times with the complete medium and placed in the wells for culture. T cell proliferation was assessed at day-4 of culture by flow cytometry.

Analysis of apoptosis and viability

The viability of cells was analyzed by labelling with Fixable Viability Dye eFluorTM 506 (FVD) (Invitrogen) according to the manufacturer's recommendations for 15 min at 4°C. After 2 washes in PBS, the cells were incubated with 1µl of AnnexinV FITC (Biolegend) in AnnexinV Binding Buffer (Biolegend). After 15 min incubation at room temperature and protected from light, the cells were washed with PBS and analyzed with a flowcytometer (Cytoflex, Beckman Coulter). FVD- cells were regarded as viable, FVD+ cells as dead, and FVD-Annexin V+ cells as apoptotic.

Flow cytometry analysis

After culture, cells were harvested and washed in PBS (Dulbecco Saline Phosphate Buffer, Eurobio, France), then incubated with the different fluorochrome-conjugated monoclonal antibodies (mAb) in Staining Buffer (BD Biosciences) for 20 minutes, then the cells were washed with PBS and analysed with a flow cytometer (Cytoflex, Beckman Coulter, Villepinte, France). The used mAbs were as follow: CD4-PE, CD3-APC-Alexa 750, CD19-APC Alexa 750 (Beckman Coulter) and CD8-APC (BD Biosciences, Le Pont de Claix, France).

For Bcl-2 expression, we performed surface labeling with CD4-Pacific Blue), CD3-APC-Alexa 750, (both from Beckman Coulter) and CD8-APC (BD Biosciences) for T cells and CD19-APC Alexa 750 (Beckman Coulter) for B cells. Afterwards cells were stained with PE-Bcl-2 (BD Biosciences, Le Pont de Claix, France) or the corresponding isotype controls, using the Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific).

Compensation beads were used for compensation parameters (VersaComp, Beckman Coulter). Data were analyzed with Kaluza Analysis v1.5a software (Beckman Coulter).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software). Comparisons between groups were performed with the non-parametric test for the cell populations of interest. Observed differences were considered statistical for a p value < 0.05.

Reference

1. Ko TK, Chuah CT, Huang JW, Ng KP, Ong ST. The BCL2 inhibitor ABT-199 significantly enhances imatinib-induced cell death in chronic myeloid leukemia progenitors. *Oncotarget* 2014; **5:** 9033-9038.