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## Venetoclax does not impair activated T cell proliferation

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► **To cite this version:**

Lama Siblany, Béatrice Gaugler, Nicolas Stocker, Laure Ricard, Yishan Ye, et al.. Venetoclax does not impair activated T cell proliferation. Bone Marrow Transplantation, 2021, 10.1038/s41409-021-01245-6 . hal-03166723

**HAL Id: hal-03166723**

<https://hal.sorbonne-universite.fr/hal-03166723v1>

Submitted on 11 Mar 2021

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1 **Venetoclax does not impair activated T cell proliferation**

2

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8

9 **Short title:** Venetoclax and T cells

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14 **Keywords:** venetoclax, T cells, allogeneic hematopoietic cell transplantation

15 Text word count: 1060, number of tables: 0, number of figures: 1, number of references: 10

16

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

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

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

39

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

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

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

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

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

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

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

86

### 87 **Acknowledgements**

88 The authors acknowledge the clinical teams who provided care for the study patients, and the  
89 Tumorothèque of Saint-Antoine Hospital.

### 90 **Conflicts of Interest**

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

### 96 **Authorship contributions**

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

101

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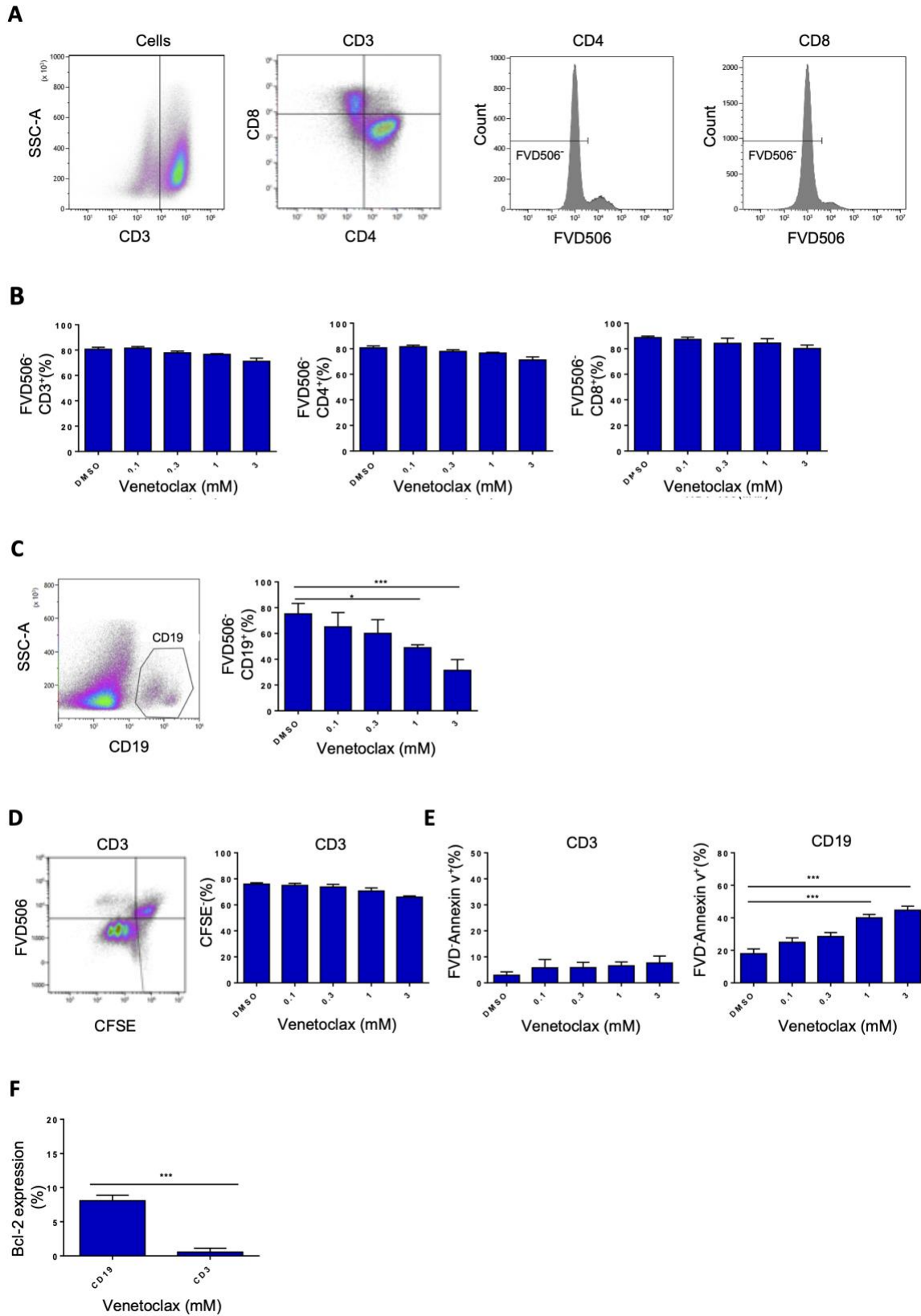
147 **Figure legend**

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

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

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

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167 **Venetoclax does not impair activated T cell proliferation**

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169 **Supplemental data**

170

171 **Material and methods**

172

173 **Venetoclax Preparation**

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

177

178 **Activation of T cells in vitro**

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

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

191

192 **Activation of B cells in vitro**

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

199

### 200 **Proliferation analysis**

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

205

### 206 **Analysis of apoptosis and viability**

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

213

### 214 **Flow cytometry analysis**

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

221 For Bcl-2 expression, we performed surface labeling with CD4-Pacific Blue), CD3-APC-Alexa  
222 750, (both from Beckman Coulter) and CD8-APC (BD Biosciences) for T cells and CD19-APC  
223 Alexa 750 (Beckman Coulter) for B cells. Afterwards cells were stained with PE-Bcl-2 (BD  
224 Biosciences, Le Pont de Claix, France) or the corresponding isotype controls, using the  
225 Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific).

226

227

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

230

### 231 **Statistical analysis**

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

235

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