Autologous T-cell activation fosters ABT-199 resistance in chronic lymphocytic leukemia: rationale for a combined therapy with SYK inhibitors and anti-CD20 monoclonal antibodies

Leukemic B cells from chronic lymphocytic leukemia (CLL) patients survive and proliferate within lymphoid tissues in contact with activated T cells, myeloid cells and by receiving signals through the B-cell receptor (BCR). ABT-199 (venetoclax) is a potent, selective inhibitor of B-cell lymphoma 2 (BCL-2) with promising clinical activity in CLL patients. ABT-199 rapidly induces apoptosis in unstimulated peripheral blood CLL cells *in vitro*, while CLL cells that have received survival signals from the microenvironment are less sensitive to the drug. In particular, signaling through the BCR or signals provided by fibroblast CD40L$^+$ and by stromal cell lines were shown to reduce the sensitivity of CLL cells to ABT-199.[1-3] In the study herein, we investigated whether resistance to ABT-199 can be conferred by autologous T-cell activation, another important survival signal from the protective niches. We first confirmed that peripheral blood leukemic cells from CLL patients are sensitive to ABT-199 *in vitro* in a dose-dependent manner (*Online Supplementary Figure S1*), while T cells (CD3$^+$ CD56$^-$), natural killer (NK) cells (CD3$^-$ CD56$^+$), and monocytes (CD14$^+$) are less sensitive to the drug (Figure 1A). Therefore, the cell death induced on CD19$^+$ cells at 24 hours of culture with 0.01μM of ABT-199 is not possible to reach, even with a hundred times more concentrated dose of ABT-199 on accessory cells (Figure 1A). Next, peripheral blood mononuclear cells (PBMC) from CLL patients were cultured on immobilized anti-CD3 monoclonal antibodies (mAbs; aCD3) to induce autologous T-cell activation. As expected, aCD3 upregulated expression of the activation markers CD69 and CD25 on CD4$^+$ and CD8$^+$ T cells at 24 hours (*data not shown*). This was not affected, or was only slightly reduced, by the presence of clinically relevant doses of ABT-199 (Figure 1B), suggesting that the drug does not significantly affect T-cell activation. Subsequently, we compared the impact of ABT-199 on the survival of unstimulated CLL cells or CLL cells that had received signals from autologous activated T cells. To this end PBMC from CLL patients were incubated with or without aCD3 for 48 hours, then ABT-199 was added to cell culture. As depicted in Figure 1C, leukemic cells cultured in the presence of activated T lymphocytes were clearly less sensitive to the drug compared to leukemic cells cultured with non-activated T cells, showing that autologous T-cell activation induced *in vitro* ABT-199 resistance in CLL cells, even at concentrations as high as 1μM. Similar results were obtained when purified leukemic cells were cultured with purified CD3$^+$ lymphocytes from the same patient (*Online Supplementary Figure S2A*). In line with this, we found a positive correlation between ABT-199 resistance induced in aCD3 cultures and the percentage of CD3$^+$ cells within the PBMC of each patient (*Online Supplementary Figure S2B*). Moreover, the inferior degree of resistance to ABT-199 in Figure 1C (see open dots in Figure 1C) was observed with samples from Patients N° 8, 15 and 17 who had very low percentages of T cells within their PBMC (see *Online Supplementary Table S1*), while no other clinical or biological association was found. Since CLL patients generally have higher absolute numbers of CD3$^+$ cells,[4] which are mainly localized within the proliferation centers of lymphoid tissues where activated T cells tend to cluster around proliferating CLL cells,[5,6] our results suggest that tissue-resident leukemic cells might not be properly targeted by ABT-199.

The activation of autologous T cells increased the expression of the activation marker CD86 in CLL cells in all of the patients evaluated (Figure 1D), and, more interestingly, it induced the upregulation of the antiapoptotic proteins MCL-1 and BCL-XL on CLL cells (Figure 1E). The fact that these proteins from the BCL-2 family are not targeted by ABT-199 may explain the resistance induced by activated T cells, which has also been suggested by recent studies analyzing other microenvironment signals.[1-3] In an attempt to restore ABT-199 sensitivity in CLL cells, we employed the BCR kinase inhibitor (BCR-KI) GS-9973 (entospletinib), which inhibits SYK and is an attractive novel therapeutic agent for CLL.[7] As shown in Figure 1F, we found that a clinically relevant concentration of GS-9973 (1μM) was able to reduce the resistance to ABT-199 induced by activated T cells.

We recently reported that GS-9973 thwarts T-cell activation in CLL patients, not only impairing the expression of CD25 and CD69 on CD3-stimulated T cells, but also reducing the proliferation and the cytokine production by T cells.[8] Moreover, when the direct effect of GS-9973 on the survival of CD19$^+$ cells from CLL patients cells was evaluated, we found that it only slightly reduced leukemic cell survival, but not in a statistically significant way, and did not particularly sensitize malignant cells to ABT-199 induced cell death (*Online Supplementary Figure S3*). Altogether, our observations suggest that the effect of GS-9973 on T-cell activation could most likely be involved in the capacity of GS-9973 to overcome the ABT-199 resistance observed in aCD3 cultures.

Our findings strengthen the role of the tumor microenvironment in the promotion of drug resistance, which might account for the progression of the disease in ABT-199 monotherapy-treated CLL patients.[10] They also encourage the combination of ABT-199 with BCR-KIs, which impair activation signals provided by the tumor microenvironment.[10] In the case of GS-9973, while we found that it overcomes the resistance to ABT-199 induced by activated T cells (Figure 1F), the reports of others showed that it prevents BCR-mediated resistance of CLL cells to the drug more efficiently than other BCR-KIs.[2]

Given that the triple combination of ABT-199 with BCR-KIs and anti-CD20 mAbs appears to be an attractive strategy (see *www.clinicaltrials.gov Identifiers: 03379051, 02950051, 02758665, 02296918* and *02427451*), we aimed to determine whether ABT-199 may affect phagocytosis of rituximab-coated CLL cells, which is a central mechanism in the anti-tumor activity of anti-CD20 antibodies.[11,12] Macrophages were exposed to different doses of ABT-199, then carboxyfluorescein succinimidyl ester (CFSE)-labeled CLL cells or rituximab-coated CFSE-labeled CLL were added to the culture. Phagocytosis was evaluated after 1 hour. Preincubation of macrophages with ABT-199 for 3 hours (*Online Supplementary Figure S4*) or 18 hours (*data not shown*) did not modify their capacity to uptake uncoated or rituximab-coated CLL cells. On the other hand, preincubation of purified CLL cells with ABT-199 significantly increased their uptake by macrophages, not only when coated with rituximab but also in its absence (Figure 2A,B). This interesting finding could be due to the exposure of "eat-me signals" on dying CLL cells which prompts macrophages to engulf them.[13] Given that phosphatidylserine (PtdSer) exposure during apoptosis is one of the best characterized "eat-me signals",[13] we evaluated Annexin V binding to PtdSer on purified CLL cells incubated, or not, with ABT-199 for 3

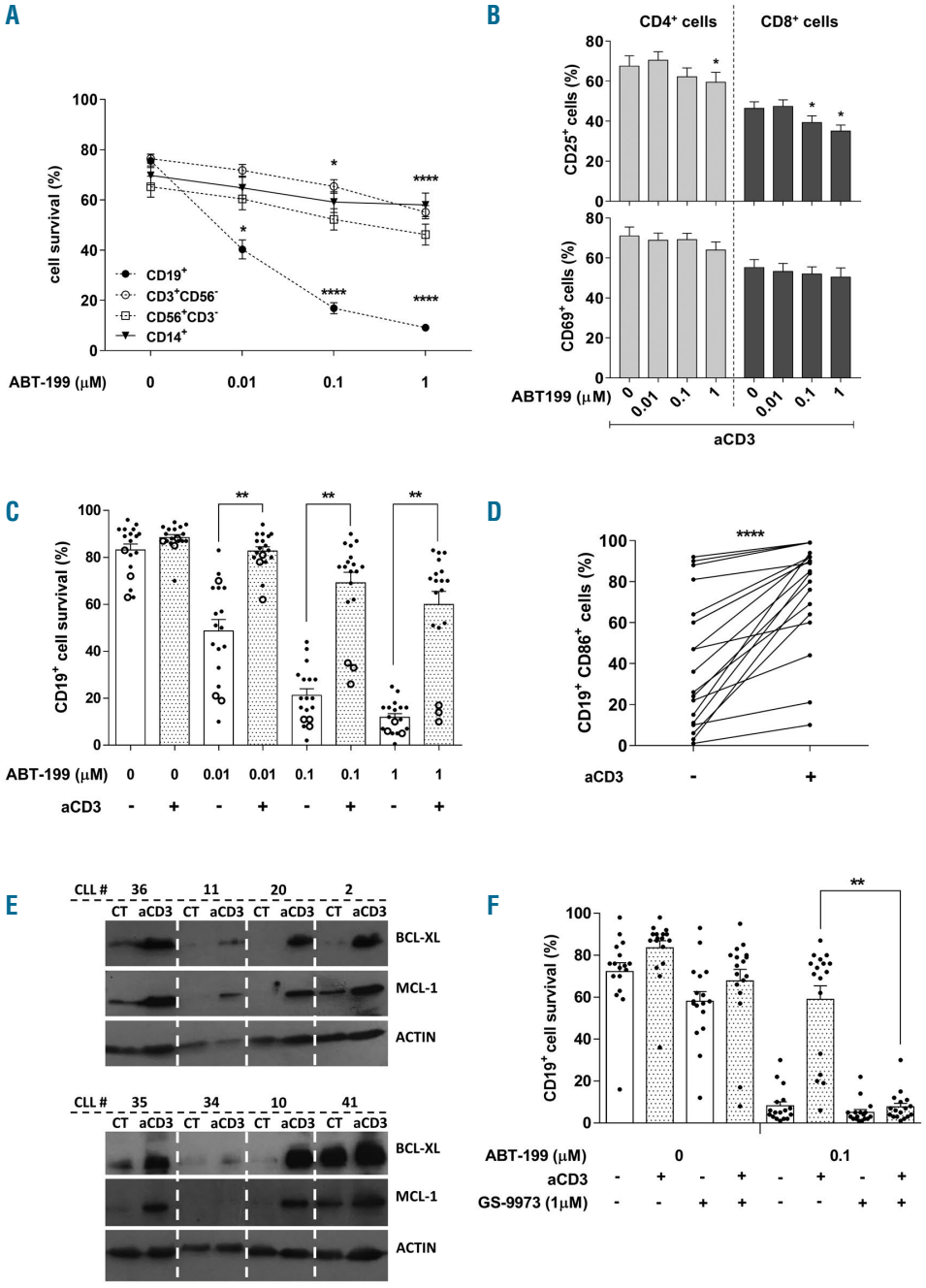

Figure 1. The activation of autologous T lymphocytes induces ABT-199 resistance in CLL which is overcome by GS-9973. A) PBMC from CLL patients (n= 30, 4 x 10^6 cells/mL) were cultured with DMSO (vehicle) or different doses of ABT-199. The survival of $CD19^+$, $CD3^+CD56^-$, $CD3^-CD56^+$and $CD14^+$ cells were evaluated daily by flow cytometric alterations of light-scattering properties (*Online Supplementary Figure S1A*) and confirmed by Annexin V-FITC assay (*Online Supplementary Figure S1B*). The figure shows the mean ± SEM of the percentage of cells within the gate of viable cells at 24 hours in control and ABT-199 cultures. Statistical analysis was performed using the Friedman test followed by Dunn's multiple comparison test. *P<0.05, ****P<0.0001, treated vs. control. B) PBMC from CLL patients (n=15, 4x10^6 cells/mL) were cultured with aCD3 (50 ng/well, 48-well plate) or the corresponding isotype control (*data not shown*) for 24 hours with or without ABT-199. Then, the expression of CD69 and CD25 on $CD4^+$ and $CD8^+$ T cells were evaluated by flow cytometry. The figure shows the mean ± SEM of the percentage of $CD4^+$ or $CD8^+$ cells expressing CD25 or CD69 in each condition. Statistical analysis was performed using the Friedman test followed by Dunn's multiple comparison test,*P<0.05, treated *vs.* control. C) PBMC from CLL patients (4 x 10^6 cells/mL) were cultured with aCD3 (50 ng/well, 48-well plate) or the corresponding isotype control for 48 hours. Then, ABT-199 or DMSO were added to the cultures for another 24 hours. The survival of $CD19^+$ cells was evaluated as mentioned above. The graph shows the mean ± SEM of the percentage of $CD19^+$ cells within the gate of viable cells and the results obtained with each CLL patient (n=18) are also shown. Open circles highlight CLL patients with less than 1% of T cells within PBMC (Patients #8, #15 and #17 of *Online Supplementary Table S1*). Statistical analysis was performed using the Friedman test followed by Dunn's multiple comparison test, **P<0.01. D) PBMC from CLL patients (n=19, 4x10^6 cells/mL) were cultured with aCD3 or the isotype control (control cultures) for 48 hours. The expression of the activation marker CD86 on CLL cells ($CD19^+$) was evaluated by flow cytometry. The figure shows the percentage of $CD19^+CD86^+$ cells of each patient in control and aCD3 cultures. Statistical analysis was performed using the Wilcoxon matched pair test, ****P<0.0001. E) PBMC from CLL patients (n=8, 4x10^6 cells/mL) were cultured with or without aCD3 for 48 hours. Next, BCL-XL and MCL-1 expression on purified CLL cells were evaluated by Western Blot as detailed in the *Online Supplementary Materials and Methods*. The figure shows the expression of BCL-XL, MCL-1 and ACTIN in control and aCD3 cultures. F) PBMC from CLL patients (4x10^6 cells/mL) were cultured with or without aCD3, in the presence or absence of GS-9973 (1 μM) for 48 hours. Then, ABT-199 was added to the cultures for another 24 hours. $CD19^+$ cell survival was evaluated as mentioned above. The figure shows the mean ± SEM of the percentage of $CD19^+$ cells within the gate of viable cells, and the results obtained with each CLL patient (n=18) are also shown. Statistical analysis was performed, using the Friedman test followed by Dunn's multiple comparison test, **P<0.01. CLL: chronic lymphocytic leukemia.

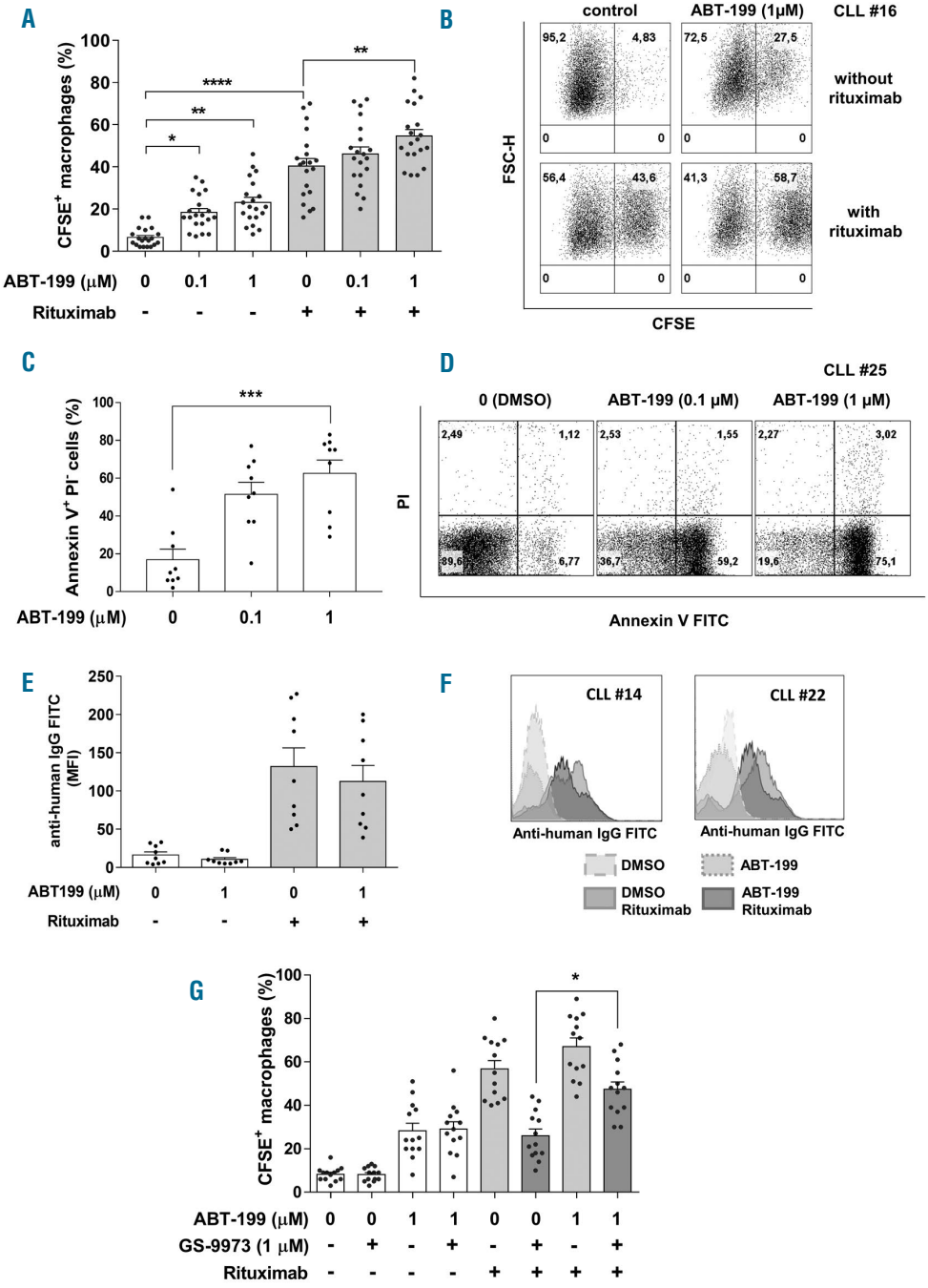

Figure 2. ABT-199 enhances CLL cells phagocytosis by macrophages. A-B) Purified CFSE-labeled CLL cells (n=20) were cultured for 3 hours with ABT-199 or DMSO and then coated or not with rituximab (50 μg/mL). The phagocytosis assay was performed as detailed in the *Online Supplementary Materials and Methods* with macrophages obtained by culturing monocytes from healthy donors PBMC in complete medium with MCSF (50 ng/mL) for 5 days. The phagocytosis was evaluated by flow cytometry after 1 hour of culture when macrophages were trypsinized. The bars in Figure A show the percentage of macrophages (determined by morphology in the FSC-H and SSC-H dot plot) that have taken up CFSE-labeled CLL cells. Statistical analysis was performed using the Friedman test followed by Dunn's multiple comparison test.*P<0.05, **P<0.01, ****P<0.0001. Representative dot plots showing FSC-H and CFSE with the percentage of macrophages in each quadrant are shown in Figure B. C-D) PBMC from CLL patients were cultured with ABT-199 or DMSO for 3 hours. PtdSer exposure induced by ABT-199 was evaluated by Annexin-V binding using Annexin-V FITC and PI. Figure C shows the mean ± SEM of the percentage of Annexin V$^+$ PI- cells in each condition, and the results obtained with each patient (n=9) is also shown. Statistical analysis was performed using the Friedman test followed by Dunn's multiple comparison test, ***P<0.001. Representative dot plots showing the Annexin V-FITC binding and PI staining of control and ABT-199 cultures, while the percentages of cells in each quadrant are shown in Figure D. E-F) PBMC from CLL patients treated or not with ABT-199 (1 μM) for 3 hours were then coated, or not, with rituximab and the antibody binding was evaluated by flow cytometry using anti-human IgG FITC. Figure E shows the mean ± SEM of the mean fluorescence intensity (MFI) of anti-human IgG FITC in control and ABT-199 cultures. The results obtained from each patient (n=9) is also shown. Statistical analysis was performed using the Friedman test followed by Dunn's multiple comparison test. Representative histogram showing rituximab binding on CLL cells treated or not with ABT-199 for 3 hours are depicted in Figure F. G) Purified CFSE-labeled CLL cells were cultured for 3hours with ABT-199 or DMSO and then coated, or not, with rituximab. The phagocytosis assay was performed as mentioned above, with macrophages pre-incubated 30 min with or without GS-9973 (n=13). Statistical analysis was performed using the Friedman test followed by Dunn's multiple comparison test, *P<0.05. CLL: chronic lymphocytic leukemia; DMSO: dimethyl sulfoxide.

hours. Results from Figure 2C confirm increased Annexin V binding in ABT-199-treated CLL cells. Representative dot plots are shown in Figure 2D. By contrast, rituximab binding to CLL cells was unaffected or even seemingly reduced by the drug (Figure 2E). Representative histograms of rituximab binding are shown in Figure 2F. The enhanced phagocytosis of rituximab-coated ABT-199-treated CLL cells might account for the substantial benefit showed in CLL patients treated with ABT-199 and rituximab compared with ABT-199 monotherapy.[14]

Finally, to test whether the stimulatory effect of ABT-199 on phagocytosis also functions in the presence of GS-9973, CLL cells were preincubated with or without ABT-199, as mentioned above, and phagocytosis was performed with macrophages treated, or not treated, with GS-9973.[8] We found that GS-9973 does not modify the uptake of uncoated CLL cells by macrophages (Figure 2G). Moreover, we confirmed our previous data, showing that GS-9973 reduced phagocytosis of rituximab-coated CLL cells,[8] and found that it also impairs the phagocytosis of ABT-199-treated CLL cells coated with rituximab. However, ABT-199 fostered uptake of rituximab-coated CLL cells in the presence of GS-9973 (Figure 2G).

In conclusion, the resistance observed *in vitro* when CLL cells were cultured with autologous activated T cells suggests that leukemic cells from the supportive microenvironment might not be properly targeted by ABT-199 monotherapy. Our results encourage the combination of the drug with a BCR-KI, such as GS-9973, which overcame the resistance induced by activated T cells and other microenvironment signals.[2] The combination with anti-CD20 mAbs could also be useful as ABT-199 enhanced phagocytosis of rituximab-coated CLL cells, even in the presence of GS-9973.

Esteban Enrique Elías,[1] María Belén Almejún,[1,2] Ana Colado,[1] Gregorio Cordini,[1,3] Maricef Vergara-Rubio,[1] Enrique Podaza,[1] Denise Risnik,[1,4] María Cabrejo,[5] Horacio Fernández-Grecco,[5] Raimundo Fernando Bezares,[6] María del Rosario Custidiano,[7] Julio César Sánchez-Ávalos,[7] Ángeles Vicente,[8] Gonzalo Martín Garate,[8] Mercedes Borge,[1,4] Mirta Giordano[1,4] and Romina Gamberale[1,4]

[1]Laboratorio de Inmunología Oncológica, Instituto de Medicina Experimental (IMEX)-CONICET-Academia Nacional de Medicina (ANM); [2]Departamento de Fisiología, Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires; [3]Hospital de Clínicas José de San Martín, Universidad de Buenos Aires; [4]Departamento de Microbiología, Parasitología e Inmunología, Facultad de Medicina, Universidad de Buenos Aires; [5]Sanatorio Municipal Dr. Julio Méndez; [6]Hospital General de Agudos Dr. Teodoro Álvarez; [7]Instituto Alexander Fleming and [8]Hospital Alemán, Buenos Aires, Argentina

Acknowledgments: the authors would like to thank María Tejeda for technical assistance and Federico Fuentes for confocal microscopy support and advice. We also thank the CLL patients who participated in the study.

Funding: GR receives a scientific research grant from Janssen, BRF and GR receive compensation as speakers for Janssen. GR receives compensation as speaker and as a consultant for Roche and Bristol Myers Squibb. BRF and CMR receive compensation as member of Advisory Board for Abbvie. The remaining authors declare no competing financial interests.

Correspondence: rominagamberale@gmail.com
doi:10.3324/haematol.2018.188680

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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