Pivekimab sunirine in blastic plasmacytoid dendritic cell neoplasm: assessing spatial response and unraveling resistance mechanisms

Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is an aggressive malignancy, which often manifests with skin lesions in addition to lymph node, blood, and bone marrow involvement.[1] Standard-of-care treatments for BPDCN patients are intense chemotherapy or a CD123-targeting drug, tagraxofusp (Elzonris®).[2] Although many patients initially respond to standard-of-care therapies, the majority of patients will eventually relapse.[3] Overexpression of CD123 is a hallmark of BPDCN thus making this antigen an attractive target.[4] Pivekimab sunirine (PVEK) is an antibody-drug-conjugate (ADC) comprised of a humanized anti-CD123 monoclonal antibody conjugated to a payload *via* a cleavable linker.[4,5] PVEK is currently being tested for the treatment of BPDCN patients as monotherapy and, as a triple therapy in combination with azacitidine and venetoclax for the treatment of acute myeloid leukemia (AML) patients.[6] Preclinically, PVEK has demonstrated potent activity against BPDCN cells[4] and importantly, showed a favorable safety profile and promising efficacy in BPDCN patients.[7] However, resistance mechanisms are often described for targeted therapies in oncology, such as ADC. Among them, a downregulation of the expression of the targeted antigen has been reporter by Loganzo *et al*., in HER2$^+$ breast cancer for example.[8] Modification of transport proteins, such as upregulation of ABC transporters was shown to decrease ADC intracellular concentration, leading to a decrease of its cytotoxic action.[9] The goal of this study was to investigate spatial response to PVEK *in vivo* in order to identify potential sanctuary organs that could lead to relapse and further resistance development. All animal procedures were carried out in accordance with the guidelines for animal experimentation (Veterinary Services for Animal Health and Protection, issued by the Ministry for Agriculture, Paris, France). We also developed a resistant BPDCN model to PVEK to anticipate the potential mechanism involved in resistance to the ADC and prevent it from happening.

The efficacy and specificity of PVEK were first assessed *in vitro* by flow cytometry (FC) on a BPDCN cell line called GEN2.2 after 24 hours (h), 48 h and 72 h of treatment (*Online Supplementary Figure S1A-C*). GEN2.2 cell line presents high expression of CD123 (Figure 1A) as well as a mutation of *TP53* (*Online Supplementary Figure S1D*), known to be associated with a worse prognosis in AML[10] rendering this model a promising tool to evaluate our drug. The ADC presents a half eliminatory concentration value (EC_{50}) between 1.3 pM and 4 pM after 72 h of treatment (*Online Supplementary Figure S1C*). Shorter treatment times (24 h and 48 h) show a similar pattern, with a reduction of the viability in a time- and dose-dependant manner (*Online Supplementary Figure S1A, B*). The payload alone showed a lower potency against GEN2.2 (EC_{50} between 12 pM and 36 pM), while the non-targeting antibody failed to reduce the cell viability even at high concentrations and long-time of exposure (EC_{50} not reached). Previous studies in AML and acute lymphoblastic leukemia (ALL) models also showed an *in vitro* efficacy of PVEK on CD123-expressing cell lines with half maximal inhibitory concentration (IC_{50}) in the picomolar range, similarly to our data.[5,11] Treatment with PVEK *in vivo* in a cell line xenograft model, using GEN2.2 cells, regardless of the concentration used, showed a controlled tumor burden compared to the vehicle-treated group (*Online Supplementary Figure S1E*). Similarly, survival of mice treated with PVEK (both concentrations) was significantly increased compared to the vehicle group (median survival of 45 days and 17 days respectively) (*Online Supplementary Figure S1F*), as previously shown in different AML mice models.[5]

To assess spatial response to pivekimab sunirine, we utilized a bioluminescent (BLI) GEN2.2-luc model. Tumor burden was significantly lower at day 16 for the treated group accompanied by a significant increased survival compared to the vehicle group (median survival of 42 day and 22 days respectively) (Figure 1B, C). On day 16 for the vehicle-treated group and day 30 for the PVEK-treated group, a separate set of mice (N=3 per group) was euthanized to harvest organs and assess tumor infiltration and response to PVEK treatment in each organ (Figure 1E). The vehicle group showed the presence of tumor cells in the bone marrow, spleen, lung and liver. PVEK-treated mice showed no sign of tumor cells in any of the organs in BLI evaluation. Hematoxylin-eosin saffron (HES) staining confirmed the elimination of tumor cells in the lung and the liver while few remaining tumor cells were observed in the spleen and the bone marrow. These few cells could be resistant cells and be the cause of relapses. Therefore we went on to evaluate potential mechanisms of resistance to PVEK. The brain did not show any signs of tumor infiltration by BLI evaluation for either of the groups (Figure 1F). However, HES staining revealed an infiltration of the brain tissue by the BPDCN cells in the vehicle-treated group. This infiltration was lower in the brain of the PVEK-treated group (Figure 1F). Very early on, the central nervous system (CNS) was considered a sanctuary for the persistence of BPDCN blasts. Indeed, in a retrospective study of 23 patients, Feuillard *et al*. described patients with CNS localization at diagnosis, but also five patients with CNS localization at relapse.[12] For further studies it may be of interest to determine whether PVEK can cross the blood-brain barrier and can kill BPDCN cells in the brain

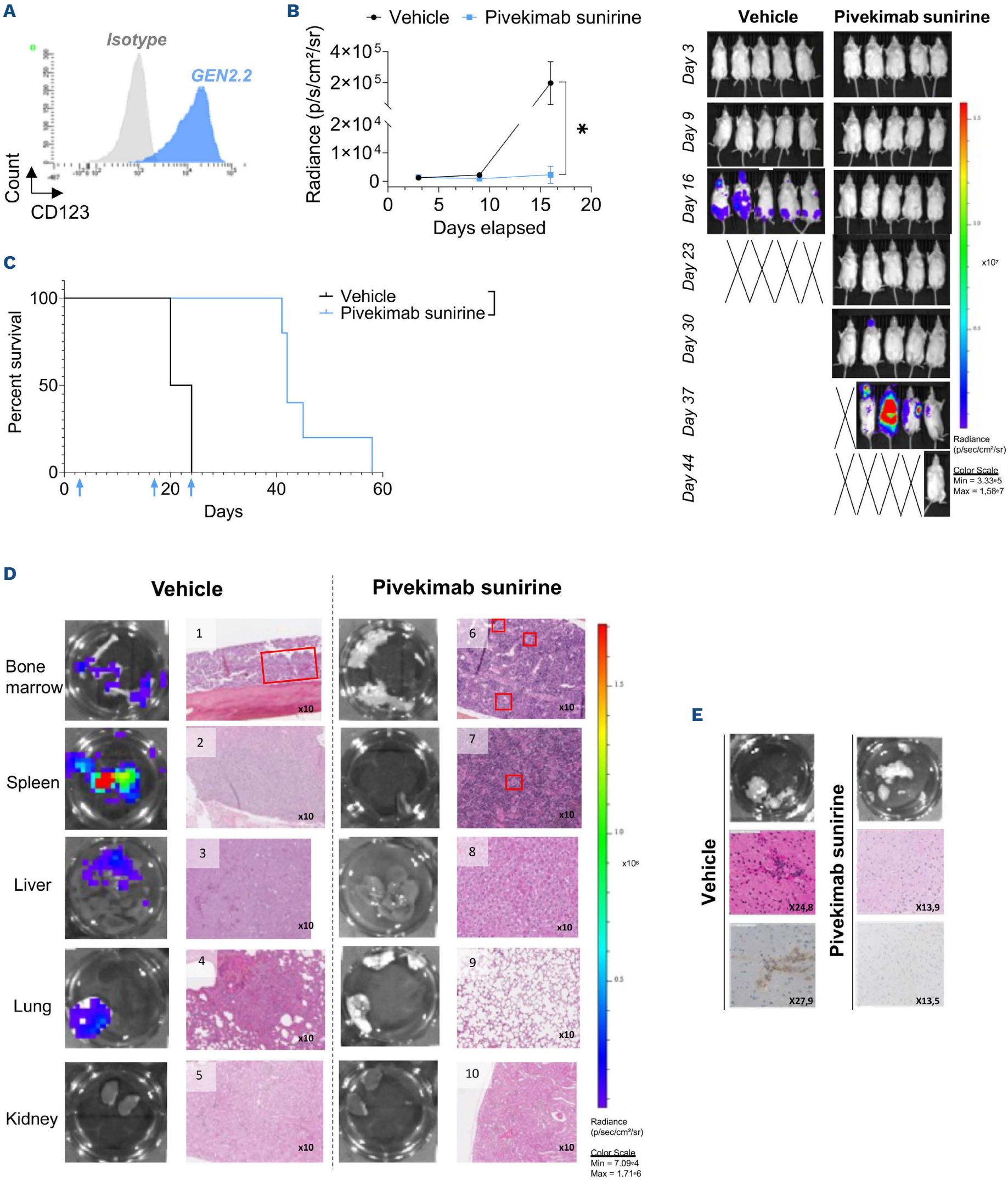


Figure 1. *In vitro* and *in vivo* spatial efficacy of pivekimab sunirine on blastic plasmacytoid dendritic cell neoplasm model. (A) Representative flow cytometry (FC) histogram of CD123 staining on GEN2.2 cell lines. (B) GEN2.2-luc-bearing mice were treated once a week for 3 weeks with pivekimab sunirine (PVEK) (intravenously). Tumor burden was monitored weekly by bioluminescent (BLI) measurements. A black cross indicates dead mice during the experiment. (C) Mice were monitored daily until death, or euthanized if they showed signs of suffering in accordance with animal welfare guidelines. Kaplan-Meier survival curves for all groups

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were generated from GEN2.2-luc injection, using death of the animal as the terminal event. Comparison of survival between groups is shown; **P<0.002. Overall survival analysis of mice revealed significant gain of survival for pivekimab sunirine-treated mice compared with untreated mice (N=5 mice per group). (D) BLI measurements was also performed on selected organs (spleen, bone marrow, liver, lung, kidney, and brain) after sacrifice at day 16 post GEN2.2-luc injection for vehicle-treated mice and at day 30 for PVEK-treated mice. Hematoxylin-eosin, saffron (HES) staining was performed on these organs to confirm results found with BLI measurement. Acquired with IVIS Lumina Series 3: field of view 24x24 cm; CCD camera 1,024x1,024 pixels. (E) HES and immunohistochemical (IHC) staining performed on brain collected at day 16 from vehicle-treated mice and at day 30 from PVEK-treated mice.

or prevent relapses with this CNS localization.

To get a deeper understanding of PVEK efficacy in BPDCN we have developed a resistant model from a BPDCN cell line. We cultured sensitive GEN2.2 cells with increasing concentrations of PVEK. After eight cycles of treatment (GEN2.2 R^{Dose8}) a reduction in sensitivity to PVEK was observed with reduction in viability of less than 40% only at the highest concentration (*Online Supplementary Figure S2A*). CD123 expression was assessed on these cells, and FC analysis showed a decrease in CD123 expression on resistant cells (*Online Supplementary Figure S2B*). In resistant cell line GEN2.2 R^{Dose8}, we sorted three fractions: (i) cells expressing the most CD123, (ii) cells expressing the least CD123 and (iii) cells expressing moderate CD123. The fraction with the lowest expression was the most resistant, with 92±5.4% viability compared to the fraction with the highest expression with 63.5±13% viability, after treatment at the highest dose (3,000 pM) (*Online Supplementary Figure S2C*). FC showed the different fractions: high, medium and low for CD123 expression (*Online Supplementary Figure S2D*). The relative fluorescence intensity (RFI) of sensitive GEN2.2 was significantly higher (25.4) than the three fractions of GEN2.2 R^{Dose8} cells (low: 1.7; medium: 2.6; high: 5.7) (*Online Supplementary Figure S2D*). The exposure of the cells to PVEK was continued and after 12 cycles of treatment (GEN2.2 R^+) complete insensitivity to PVEK was observed even at the highest concentrations (95.6±1.6 % viability) (Figure 2A). The evaluation of the sensitivity of those cells to PVEK confirmed that we successfully established a resistant cell line to this ADC. CD123 expression of GEN2.2 R^+ was close to isotype control and the RFI of this cell line is negative (1.5±0.3) compared to GEN2.2 (22.4±4.4) (Figure 2B). Significant decreased expression of CD123 was further confirmed by FC using another clone, which does not target the same area of the FC as PVEK (G4723A) (*Online Supplementary Figure S2E*). Intracellular staining by FC, of CD123 also showed a significant reduction of the RFI by GEN2.2R^+ compared to GEN2.2 (0.9±0.0 and 46.2±0.0, respectively) (*Online Supplementary Figure SF*).

To assess the molecular mechanism contributing to the establishment of BPDCN resistance to PVEK, we performed RNA sequencing of GEN2.2 and GEN2.2 R^+ cell lines. Expectedly, we observed a clear segregation of these two entities (Figure 3A). We further found 2,287 differentially expressed genes between GEN2.2 and GEN2.2 R^+, of which 1,491 and 796 are respectively upregulated and downregulated in GEN2.2 (Figure 3B). Our results showed an antigen loss (CD123) at protein levels but not RNA levels. Some studies have shown the acquisition of a mutation under treatment, leading to a lower affinity between the protein and the antibody.[13] Truncated forms of the antigen are another potential mechanism of resistance.[14] Further investigations are thus needed to confirm which mechanism could explain why the CD123 decrease observed at the protein level is not seen at RNA level. We also observed an upregulation of *ABCB1* expression

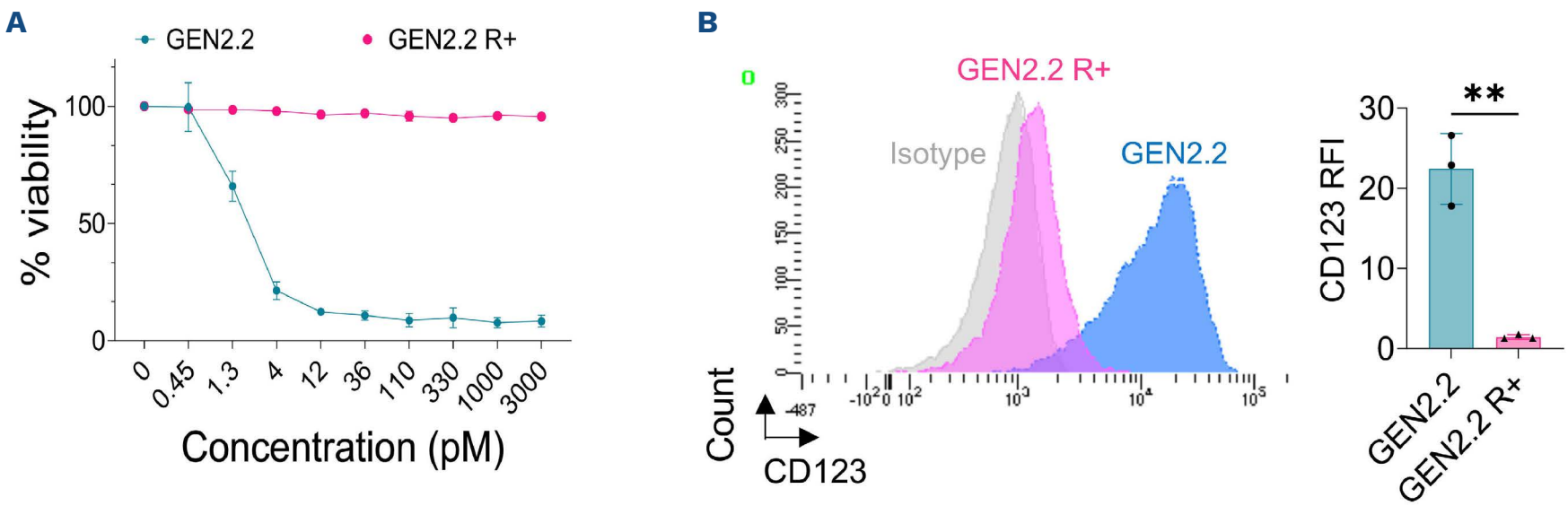
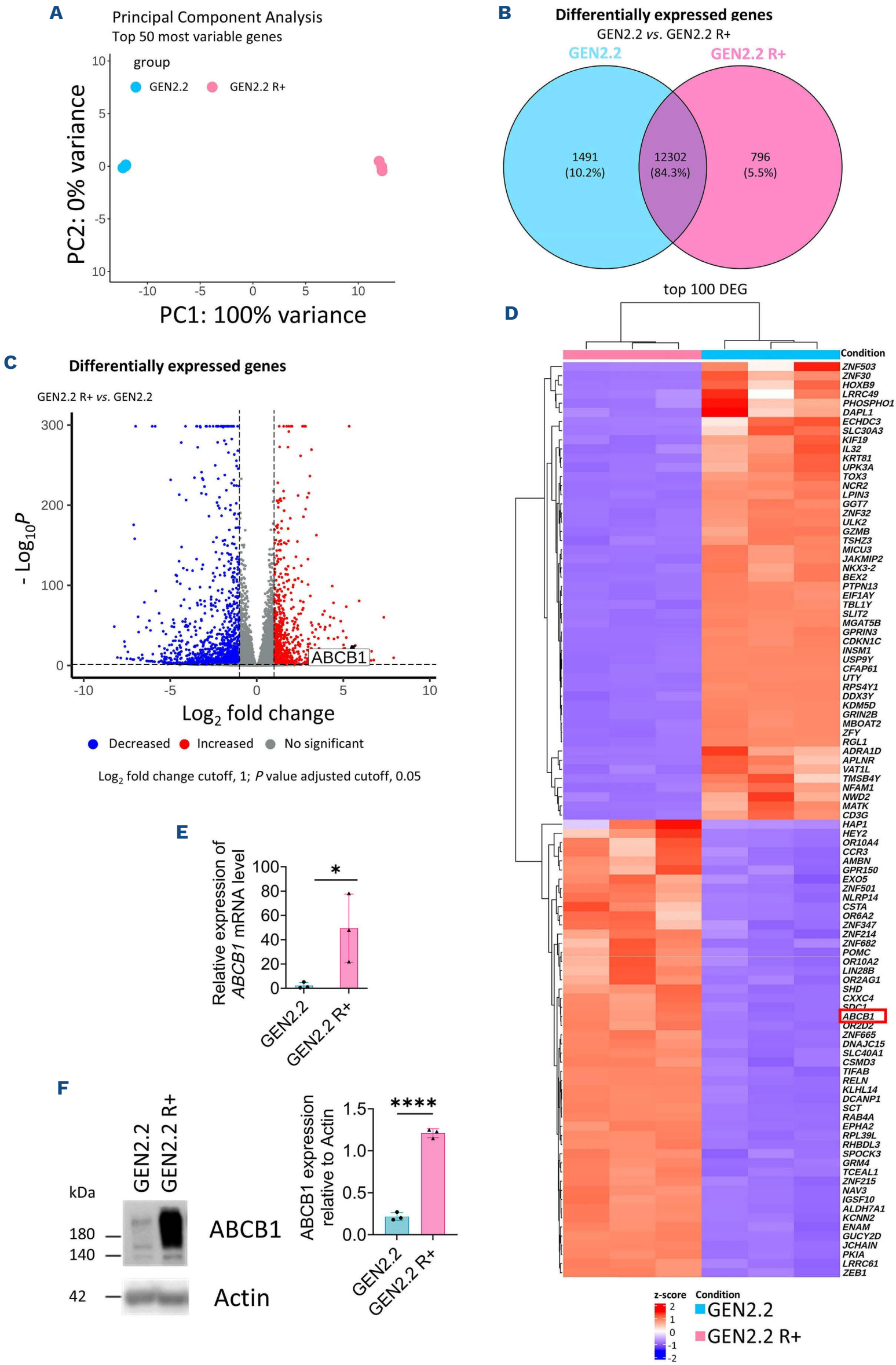


Figure 2. Phenotypic characterization of established blastic plasmacytoid dendritic cell neoplasm cell line resistant to pivekimab sunirine. (A) GEN2.2 cells were cultured in the presence of chronic pivekimab sunirine (PVEK) exposure (addition every 72 hours [h], increasing concentrations (blue) over time). Acquired resistance was subsequently assessed by flow cyotmetry (FC) after 72 h of treatment with PVEK at different concentration (0.45 to 3,000 pM) (N=3). Resistant cells to PVEK are named GEN2.2 R^+ (pink). (B) Representative FC histogram of CD123 staining on GEN2.2 (blue) cells and GEN2.2 R^+ (pink) cells compared to isotype control (left) and all experiments of CD123 cell surface expression on GEN2.2 *versus* GEN2.2 R^+ (N=3; **P<0.002) (right).



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Figure 3. Transcriptomic analysis of established blastic plasmacytoid dendritic cell neoplasm cell line resistant to pivekimab sunirine reveals modified ABCB1 expression. (A) Principal component analysis (PCA) of GEN2.2 (blue) and GEN2.2 R^+ (pink). Resistant cells to pivekimab sunirine (PVEK) are named GEN2.2 R^+.(B) Differentially expressed genes (DEG) between GEN2.2 *versus* GEN2.2 R^+. (C) Volcano plot of protein encoding genes in GEN2.2 *versus* GEN2.2 R^+ showing the relationship between fold change (log2 fold change [FC]; horizontal axis) and significance (-log10 [*P* value]; vertical axis), respectively. *ABCB1*: log2FC=5,69; adjusted *P* value=$1.13x10^{-25}$. (D) The list of top 100 DEG in GEN2.2 (blue) and GEN2.2 R^+ cells (red). (E) The mRNA expression of *ABCB1* was assessed by quantitative polymerase chain reaction on cells treated with PVEK. Results are reported as relative expression to GEN2.2 (N=3; **P*<0.05). (F) Western blot analysis realized with specific anti-ABCB1 transporters primary antibodies. Upper panel: representative blot of 3 independent experiments. Lower panel: quantification of expression intensity of ABCB1 by Image J software. β-actin was used as a loading control (N=3; *****P*<0.0001).

which is part of the top 100 differentially expressed genes between GEN2.2 and GEN2.2 R^+ (Figure 3C, D). The significant upregulation of ABCB1 transporters was confirmed at mRNA level quantitative polymerase chain reaction with significantly higher expression on GEN2.2 R^+ compared to GEN2.2 (49.5±28 compared to 2.25±2.5. respectively) (Figure 3E) and further at the protein level by western blot (Figure 3F). Upregulation of ABC transporter expression was reported several times in cancer resistance to chemotherapies and ADC.[9] Yu *et al*. showed in a non-Hodgkin lymphoma model made resistant to ADC targeting CD22 and CD79b that upregulation of P-glycoprotein (P-gp)/ABCB1 was the major driver of the resistance to the ADC payload.[15] They further showed that changing the payload while keeping the same targeted antigen could also reverse the resistance acquired by the tumor cells.[15]

Overall, our data provide an additional BPDCN model showing efficacy of PVEK as well as detailed information for the first time of spatial response to this ADC. Our resistant model also helps us to identify potential mechanism of resistance that could be developed by patients upon PVEK treatment. Further experiments are necessary to confirm the described mechanism in patients relapsing and also to evaluate strategies to overcome the resistance. Antigen loss and efflux pumps are known resistance mechanisms observed in ADC and it is critical to monitor these components in PVEK clinical trials. Further experiments are thus necessary to confirm the described mechanism and to evaluate strategies to overcome the resistance.

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No conflicts of interest to disclose.

Contributions

MB and MP performed the experiments. MB, IB and MP analyzed data. MP and SB provided help with flow cytometry analysis. MB and MP performed statistical analysis. KVM performed the western blot analysis. FM and CB analysed the HES and IHC staining. BJ, XR, SB, MF, FR, FGO, OA and FAD provided guidance and expertise in their respective areas of study. MB, MP and FAD wrote the manuscripts. FGO and OA commented on the manuscript. FGO and FAD supervised the research. All authors provided input and edited and approval the final version of the manuscript.

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Data-sharing statement

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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