Cyclophosphamide and vinorelbine activate stem-like CD8+ T cells and improve anti-PD-1 efficacy in triple-negative breast cancer

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Running Title: Cyclophosphamide and vinorelbine activate stem-like T-cells
Abstract

Checkpoint inhibitors (CI) instigate anticancer immunity in many neoplastic diseases, albeit only in a fraction of patients. The clinical success of cyclophosphamide (C)-based haploidentical stem-cell transplants indicates that this drug may re-orchestrate the immune system. Using models of triple-negative breast cancer (TNBC) with different intratumoral immune contexture, we demonstrate that a combinatorial therapy of intermittent C, CI, and vinorelbine (V), activates antigen presenting cells (APC), and abrogates local and metastatic tumour growth by a T-cell-related effect. Single-cell transcriptome analysis of >50,000 intratumoral immune cells after therapy treatment showed a gene signature suggestive of a change resulting from exposure to a mitogen, ligand, or antigen for which it is specific, as well as APC-to-T-cell adhesion. This transcriptional program also increased intratumoral tcf1+ stem-like CD8+ T cells and altered the balance between terminally and progenitor-exhausted T cells favoring the latter. Overall, our data support the clinical investigation of this therapy in TNBC.

Statement of Significance

A combinatorial therapy in mouse models of breast cancer increases checkpoint inhibition by activating antigen presenting cells, enhancing intratumoral tcf1+ stem-like CD8+ T-cells, and increasing progenitor-exhausted CD8+ T-cells.

Introduction

Checkpoint inhibitors (CIs) have shown an unprecedented clinical activity in several types of cancer, but only a fraction of patients has benefit from CIs administered as a single therapy. Moreover, in some patients the clinical responses are limited in time (1). These observations are promoting preclinical and clinical studies to improve CI efficacy by means of combinatorial therapies (2).

The clinical success of cyclophosphamide (C)-based haploidentical stem cell transplants in hematological malignancies indicates that this drug has the potential to re-orchestrate the immune system against cancer cells (3). Along a similar way, C is administered before CAR-T cell infusion to improve their clinical efficacy (4). We have previously found that low-dose, daily C, in association with V was able to improve the
preclinical efficacy of CIs anti-PD-1 and anti-PD-L1 in models of breast cancer and lymphoma (5). As randomized clinical trials have recently indicated that the addition of CIs to other chemotherapy drugs such as taxanes (T), doxorubicin (D), or platinum (P) might be beneficial in triple negative breast cancer (TNBC) patients (6–10), we designed the present study in two preclinical, immunocompetent models of TNBC (4T1 and EMT6) to investigate whether C and V, in different schedules and doses, could be more effective than T, D, or P.

The first series of preclinical studies reported here have clearly indicated that intermittent, medium-dosage C (140 mg/kg, C140), a dosage already known to have potent effects on the immune system (11), was significantly more effective than lower, continuous doses of the same drug (20 mg/kg, C20), and that intermittent C140, in association with V and CIs, were significantly more effective than combinatorial regimens of CIs plus T, D, or P. Thus, we investigated by means of neutralizing monoclonal antibodies, single-cell transcriptome analysis and flow cytometry what immune cells were involved in these anti-TNBC mechanisms and how these drugs reshaped the circulating and intratumoral immune cell landscape in cancer-bearing mice.

A recent meta-analysis from clinical trials involving anti-PD-1 and anti-PD-L1 CIs indicated that anti-PD-1 had a favorable survival outcomes and a safety profile comparable to that of anti-PD-L1 (12). For this reason, we are showing here only the results obtained in our clinical models with the anti-PD-1 CI. At the present time we have no evidence that anti-PD-L1 can be more effective of anti-PD-1 in these models.
Materials and methods

Cell culture

The 4T1 and the EMT6 TNBC cell lines were purchased from ATCC, expanded and stored according to the producer’s instructions. Cells were tested and authenticated by the StemElite ID System (Promega). Cells were tested every six months for Mycoplasma by means of the ATCC Universal Mycoplasma Detection Kit 30–1012, cultured for no more than two weeks and used for no longer than 15 passages.

Cell line transduction

The 4T1 and EMT6 cell lines were stably transduced with lentiviral vector expressing stably luciferase under the control of CMV promoter: pLenti CMV Puro LUC (w168-1) (Addgene plasmid # 17477), carrying puromycin resistance as selective marker. To infect 4T1 and EMT6 cell lines, 293T cells were co-transfected with 10 µg of lentiviral vector, 3 µg PMD2G envelope plasmid, 2.5 µg of REV packaging plasmid and 5 µg of RRE transfer plasmid using Calcium Phosphate transfection system. After 16 hours, media was removed and replaced with 5 ml of fresh medium. Viral supernatant was then collected at 24 and 48 hours and added directly to 4T1 and EMT6 cell lines plated at 1*10^5 supplemented with 8 µg/ml of polybrene (Merck). Infection was performed for 3 hours at 37°C twice in a day for two separate days. After 48 hours from first cycle of infection, infected cells were selected adding 1.5 µg/ml (for 4T1 cell line) and 2 µg/ml (for EMT6 cell line) puromycin (Merck) to the medium. Selection was carried out for 72h, until not infected control cells were dead.

In vivo studies

Experiments involving animals were approved by the Italian Ministry of Health and have been done in accordance with the applicable Italian laws (D.L.vo 26/14 and following amendments), the Institutional Animal Care and Use Committee and the institutional guidelines at the European Institute of Oncology. In vivo studies were carried out in 6-8 weeks old immune-competent BALB/cOlaHsd female mice (Envigo) in mouse facilities at the European Institute of Oncology–Italian Foundation for Cancer Research (FIRC) Institute of Molecular Oncology (IEO–IFOM, Milan, Italy) campus. To generate syngeneic models of TNBC in BALB/c, 2×10^5 4T1-LUC, and 5×10^5 EMT6-LUC were injected in the mammary fat
pad as we previously described (5). Tumour growth was monitored weekly using In Vivo Imaging System (IVIS) (PerkinElmer). Briefly, mice were IP injected with 150 mg/kg of XenoLight D-Luciferin – K+ Salt Bioluminescent Substrate (PerkinElmer). After 10 minutes, animals were anaesthetized with isofluorane apparatus and images acquired using Living Image Software (PerkinElmer) (Supplementary Figure S1A and B). Tumour dimension was finally assessed using ImageJ software, by converting pixel intensity into centimeters and determining finally tumour mass sphere volume in mm³.

**TNBC metastatic models**

TNBC resection was performed 28 (for all EMT6 experimental groups and for 4T1 control, anti-PD-1, D+ anti-PD-1, T+ anti-PD-1, P+ anti-PD-1 experimental groups) or 70 (for C140-treated experimental 4T1 tumour) days after tumour implant as we previously described (5,13,14). Fifteen days after mastectomy, mice were sacrificed by carbon dioxide inhalation and lung tissues were removed. To confirm the presence of metastases, sections were cut and stained with haematoxylin and eosin (H&E), as previously described (5). In brief, lungs were fixed in 4% phosphate-buffered formalin and embedded in paraffin. Five micrometres thick sections of lungs were made, and slides were counterstained with H&E for the detection of metastases. Images were acquired with a ScanScope XT scanner (Leica) and analyzed with Aperio Digital Pathology software (Leica). To assess the metastases in the lungs, ImageJ software was used. Areas occupied by the metastases was divided to total lungs’ volume to assess the percentage of metastasis in the lungs.

**In vivo therapy**

Tumour-bearing mice ($n = 5$ per study arm) were treated with either vehicle or with different drugs used as single agents or in combination. Drug dosages used in this study are the gold-standard chemotherapeutic drugs for TNBC and doses were based on literature data associated with no or acceptable toxicity, as well as no significant changes in mouse weight. C was used at two different doses: 20 mg/kg in drinking water (C20) and 140 mg/kg (C140) in intraperitoneal injection (IP) as already shown in (5,11). V was used at 9 mg/kg as we have previously shown in (5). P was used at 0.25 mg/kg (15), T was used at 20 mg/kg (16), D was used at 2.9 mg/kg (15). Chemotherapeutic drugs were dissolved in PBS and administrated, once a week, every 6 days, for 3 weeks by IP injection. Mouse monoclonal
PD-1 targeting antibody was purchased by Bioxcell (clone RMP1-14) and was administered IP 0.2 mg/mouse every 2 days for a total of 5 doses. Monoclonal neutralizing antibodies were purchased by Bioxcell. Anti-CD4 (clone GK1.5), anti-CD8a (clone 2.43), anti-CD19 (clone 1D3), anti-CD11b (clone M1/70), anti-CD103 (clone M290) and anti-CD122 (clone TM-beta1) were administrated 300 µg/mouse twice a week for a total of 5 doses.

**Tumour dissociation and cell sorting for scRNA-seq library preparation**

Mice tumours were surgically removed after 28 days from tumour injection to generate a single cell suspension. Briefly, after mechanical dissociation, tumours were placed in culture medium (1:1 of Dulbecco’s Modified Eagle’s Medium with high glucose and Ham’s F-12 Nutrient Mixture, EuroClone) supplemented by 2 mg/mL collagenase (Merck) and 0.1 mg/mL Dnase I (Qiagen), and digested for 1–2 h at 37 °C. A single cell suspension was obtained by sequential dissociation of the fragments by gentle pipetting, to further disintegrate cell clumps, followed by filtration through a 100-μm nylon mesh. After cell suspension preparation, cells were incubated with PE-conjugated murine CD45 antibody (BD Bioscience, clone 30-F11) for 20 minutes at 4°C. Cells were then washed twice with PBS+0.2% BSA and prepared for cell sorting. Before sorting, cells were co-stained with 0.1 µg/ml 4′,6-diamidino-2-phenylindole (DAPI, Merck). CD45+DAPI- cells were sorted using FACS Fusion sorter (BD Bioscience). Post-sorting cell purity was assessed by FACS analysis (Supplementary Figure S2A and B) and purity was assessed to be higher than 90%. Cells were then counted and 5000 cells/conditions underwent scRNA-seq library preparation following 10X Genomic V2 and V3 protocol.

**Tumour dissociation for FACS analysis**

Mice tumours were surgically removed and processed to obtain cell suspension as explained in the previous paragraph. Gating strategies are summarized in Supplementary Figure S3A and B. Briefly, viable cells (negative for 7-aminoactinomycin, 7AAD, Beckman Coulter) were stained with antibodies cocktail mix containing anti-CD45, anti-CD3e, anti-CD8, anti-CD4 to identify CD3+CD8+ and CD3+CD4+T cells. Than cells were stained with anti-CD279 (anti-PD-1) and anti-Tim3 antibodies. Percentage was referred to alive lymphocytes.
Alignment and quality control analyses

After demultiplexing, reads were aligned with Cell Ranger v3.0.2 to the mm10 reference genome using the GENCODE vM16 feature annotation (17). Transcript counts from genes with the same symbol but different ENSEMBL ID were summed up, resulting in a total of 53,278 unique genes. We merged the raw transcript counts from all six treatments for each cell line using Seurat v3 (18,19). Then, cells with 500 transcripts or less, 50,000 transcripts or more, fewer than 250 expressed genes, fewer than 0.80 log10 genes per UMI (Supplementary Figure S4A and B), or more than 15% mitochondrial reads were removed from the analysis (Supplementary Figure S5A and B; Supplementary Table S1, and Supplementary Table S2). Similarly, genes expressed in fewer than 5 cells were removed from the analysis, resulting in 22,806 and 23,757 genes on 4T1 and EMT6 cell line, respectively (Supplementary Figure S6A and B). Downstream analyses, if not specifically mentioned otherwise, were carried out using Seurat v3.

Normalization, batch correction, clustering and cell cycle analysis

Transcript counts were log-normalized using the “NormalizeData()” function. Canonical correlation analysis (CCA) was used to integrate data generated with the v2 chemistry and the v3 chemistry of the 10x Genomics Single Cell 3’, respectively. First, raw transcript counts were normalized with the “SCTransform()” function separately for each data set of the two chemistry version. Then, the two data sets were integrated using the “SelectIntegrationFeatures()” (to select 3000 features for integration in each data set), “PrepSCTIntegration()”, “FindIntegrationAnchors()”, and “IntegrateData()”. Subsequently, principal component analysis (PCA) was performed on the integrated data set. The first 30 principal components were used to construct a Shared Nearest Neighbor (SNN) graph, from which clusters were calculated through the “FindClusters()” function and the resolution parameter set to “0.8”. The Uniform Manifold Approximation and Projection (UMAP) method was used for dimensionality reduction and calculated from the first 30 principal components. Cell cycle analysis was performed using the “CellCycleScoring()” function and the S- and G2M-specific genes downloaded from ensembldb package (20) (Supplementary Figure S7A and B).
Cell type assignment

Cell type annotation was carried out with the SingleR R package (21) and the “ImmGenData” reference (22), containing 253 fine labels generated from 830 microarray samples of sorted cell populations. We performed an initial assignment to the finest level possible using the fine labels and then manually grouped together the cell populations to reach a reasonable granularity that helps exploration of the dataset. The conversion of the manually assigned groups of cells can be found in Supplementary Table S2. At both levels, cells belonging to labels with less than 20 cells were assigned to the group “Others” (Supplementary Table S2). Chi-square statistics using as expected the T cell population frequencies of the control was performed, and its strength was tested with the Cramer’s V test. Chisq.test() and an in-house R functions were used to calculate the two tests respectively (Supplementary Table S3). We measured the fold change of the T cell populations in each treatment as result of the expression: Fi / Fc, in which Fi is the frequencies of the T cell population in the specific treatment while the Fc is the frequencies in the control. Correlations between the T cell fold changes were performed either with Spearman or Pearson tests according to their normality as in (23). Only the significative comparisons were plotted.

Marker genes detection and pathway enrichment analysis

Marker genes were identified through the “FindAllMarkers()” Seurat function with a Wilcoxon test. Parameters were set to only identify over-expressed genes that are expressed in at least 70% of the cells of the cell population of interest, e.g. cluster, with a log fold-change of at least 0.25 compared to all other cells. Furthermore, only genes which passed statistical testing with a p-value of 0.01 or lower were retained. Pathways enriched in the marker genes were retrieved using the “getEnrichedPathways()” function from the cerebroApp R package (24), which queries the Enrichr database (25,26). Results were filtered for pathways with an adjusted p-value of 0.05 or lower.

Gene expression evaluation

Expression values of single genes shown in violin plots represent the log-normalized counts from “RNA” assay. To represent the expression of gene sets, we either calculated a module
score with the “AddModuleScore()” function, or computed the average expression with the “AverageExpression()” function applied to the “SCT” assay.

**Gene set variation analysis (GSVA)**

In order to perform the Gene Set Variation Analysis (GSVA), we downloaded the 50 hallmark gene sets (27) using “msigdb” package(28). From those, we selected 26 pathways of interest with a specific interest for the proliferation and immune cells activation groups. Then, we removed duplicated genes within and between pathways following the procedure by Lambrechts et al. (29). Finally, for a subset of the data set containing only T cells, we estimated the GSVA score on the raw transcript counts, fit a linear model and estimated the moderate t-statistic in default mode for each combination of treatments.

**Flow cytometry**

At least 100,000 cells per sample were acquired using a 3-laser, 10-colour flow cytometer (Navios, Beckman Coulter, Brea, CA, USA). Viable cells (negative for 7-aminoactinomycin, 7AAD, Beckman Coulter) were labelled with a panel of antibodies to analyze immune cell populations. Antibodies list and gating strategies are summarized in the Supplementary Table S4. Lymphocytes and myeloid cells were characterized using state-of-the-art markers. Specifically, cells were gated for size, singlets and then by positive and negative markers: CD3+CD4+ and CD3+CD8+ T cells, CD335+ NKs, CD19+ B cells, Gr1-CD11b+CD11c+ monocytes. SSC^high^CD11b+Gr1+ granulocytes and CD11c+CD11b-Gr1- antigen-presenting cells (APC). APC were also marked with CD80 to determine their activation. For each immune cell subtype, we calculated cell absolute value by multiplying the percentage to the total number of white blood cells/µl.

**T-cell receptor analysis**
T cell receptor clonality was analyzed in the tumour infiltrate using FITC Hamster anti-mouse beta TCR kit (BD Bioscience, clone H57-597), following manufacturer’s protocol. Briefly, tumour was dissociated as shown in the previous paragraph and 5*10^5 cells were stained with antibody cocktail mix containing: 7AAD (Beckman Coulter), CD45 APC-Cy7 (BD Bioscience, clone 30 F-11), CD3 APC (BD Bioscience, clone 145-2C11), CD4 PE (BD Bioscience, clone GK1.5), CD8 PE-Cy7 (BD Bioscience, clone 53-6.7) and a specific region of VDJ beta chain rearrangement. Viable cells were gated for CD45/CD3 positivity. Then, cells were gated for CD4 and CD8 positivity, and among them we evaluated for each beta chain rearrangement the percentage of positivity. Finally, fold increase was determined by dividing the percentage of positivity in the untreated control versus each experimental condition.

**Statistical analysis and graphical representation.**

Data were expressed as means ± sem (in case of normal distribution) Normal distribution was assessed using Shapiro Wilk’s normality test. To compare two sample groups, either the Student’s t-test or the Mann-Whitney U-test was used based on normal or not normal distribution. Statistical analysis was carried out Prism 8.3.1. (GraphPad) and R(30) (URL http://www.R-project.org/). Graphical model was created using Biorender Software (https://biorender.com/).

**Data Availability**

Single-cell transcriptome data of tumour samples and codes used for their analysis are available from the corresponding author upon request.
Results

Intermittent, medium-dosage C140, was more effective than other combinatorial regimens including CIs plus low-dose C, or regular-dose T, D, or P. The association of CIs and V further increased the preclinical efficacy of C140.

We injected 5 mice per experimental group with 4T1 and EMT6 cells and treated them with chemotherapeutic agents, CI and chemotherapeutic agents combined to CI. Chemotherapeutic agents were administered every six days, while anti-PD-1 every two days for a total of 5 injections, as shown in the therapeutic scheme presented in Supplementary Figure S8A. We measured tumour growth in both models, and as shown in Figure 1 (A and B) and Supplementary Figure S8 B, C. In vivo studies in local and metastatic models using both 4T1 and EMT6 TNBC cells clearly indicated that intermittent (i.e. every 6 days), C140 was more effective than any other combinatorial regimens including CIs plus daily C20, or T, D, or P at their regular preclinical dosages.

For the EMT6 model, the triple therapy abrogated tumour growth in 28 days (p<0.05), while for 4T1-bearing mice, C140 alone abrogated tumour growth up to day 28. Therefore, for 4T1 model, we prolonged the time schedule to 70 days and we observed that the association with V and CIs (anti-PD-1) further increased the preclinical efficacy of C140 (p<0.001). Tumor resection was performed for 4T1 tumor on day 28 for control, anti-PD-1, D+ anti-PD-1, T+ anti-PD-1 and P+ anti-PD-1, and on day 70 for all C-treated groups; whilst for EMT-6 tumor, resection was performed at day 28 for all treatment groups. After tumour resection, we observed that the triple therapy abrogated metastatic tumour dissemination in the lungs in the 4T1 model (p<0.001) (Figure 1C). In the EMT6 model, we observed a reduction in metastases with the triple therapy compared to controls, even though this mouse model is known to generate less metastases (31), (Figure 1C and D and Supplementary Figure S8 D and E).

T cells are needed to control tumour growth in mice treated with C140, V and CIs.

To further demonstrate that immunity plays a crucial role in tumour eradication, we performed preclinical studies with neutralizing monoclonal antibodies targeting in separate experiments T, B, NK, and myeloid cells as shown in the therapeutic scheme presented in Supplementary Figure S9A. Neutralizing antibody efficacy was tested by FACS as shown in
the Supplementary Figure S9 B-D. In TNBC-bearing mice treated with C140, V and CIs, the combinatorial therapy demonstrated that CD3+CD8+ (in 4T1-bearing mice), and CD3+CD8+ plus CD3+CD4+ T cells (in ETM6-bearing mice) were needed to abrogate TNBC growth (p<0.05) (Figure 1 E, F). To a much lesser extent, in ETM6-bearing mice also CD11b+ myeloid cells (p<0.01), CD19+ B cells (p<0.01) and CD122+ NK cells (p<0.01) had a role in reducing TNBC growth. 

Chemotherapy induces selective modulation of circulating and intratumoural immune cell subsets and circulating T cell clones.

As we previously observed (5), during therapy the circulating landscape and the intratumoural contexture of immune cells were markedly different. Therefore, we decided to analyze the immune cell landscape in the peripheral blood by flow cytometry, as shown in Supplementary Figure S10A and B, and within the tumour by single cell RNA seq analysis (scRNA-seq).

In the peripheral blood, C140-containing regimens significantly reduced the number of circulating lymphoid and myeloid cells in both mouse models (Supplementary Figure S10A and B). As also previously reported (5), flow cytometry and single-cell transcriptome analyses confirmed that V-containing regimens were associated with significant APC activation, measured by CD80 expression (Figure 2A, B and Supplementary Figure S11). Moreover, flow cytometric immunophenotypic assessment of T-cell receptor (TCR)-V(β) repertoire within tumours suggested a significantly larger circulating CD3+CD4+ and CD3+CD8+ T cells clonal expansion in mice treated with C140 when compared to mice treated with other drugs (Figure 2C-F).

Using scRNA-seq analyses, we observed that 4T1 and ETM6 TNBC tumours were significantly different in the landscape of intratumoural immune cells: 4T1 tumours were more infiltrated by lymphoid cells, while ETM6 tumours by myeloid cells (Figure 3A and B and Supplementary Table S5). In both models single-cell transcriptome analyses of more than 50,000 intratumoural immune cells indicated that C140, V and CI treatment was associated with the largest T cell intratumoural population, with a predominant presence of CD3+CD4+ T helper cells (Th1 cells), known to promote anti-tumour immunity (32) (Figure 3A and B). To assess cell diversity, chi-square statistics and Cramer V test were applied for both the cell lines using as expected values the T cells frequencies of the control. The number of cells resulted significative different from the control for each treatment in both the
cell lines (Supplementary Table S5). Moreover, we measured the increase and the fold change of the T cell populations for each treatment compared to the control. We identified a strong correlation between the increase of T cells for PD1, Cisplatin + PD1 and triple treatments between the two lineages (Supplementary Figure S12A-F).

Single-cell transcriptome analyses showed several unique T-cell activation patterns associated C140, V and CI treatment.

As intratumoral T cells were more abundant in mice treated with C140, V and Cls, we tested the hypothesis of a phenotypic change in these T cells and performed an analysis of hallmark pathway gene signatures as in (28). We compared the pathways observed in control and after C140, V and Cl treatment and observed that this treatment increased the expression of pathways involving proliferation (i.e. myc target, G2M checkpoint) and T cell activation (i.e. Interferon alpha and gamma response, Figure 4 A, B). Our data strongly suggest that C140, V and Cl induce T cell proliferation and activation.

Moreover, we studied the expression of T cell different activating genes (Cd27, Cd28, Cd69, Cd25 and Interferon gamma among others, see Figure 4C, D), and confirmed that after C140, V and Cl these genes were more expressed in intratumoral T cells, strongly suggesting that the therapy may increase T cell activation against the tumour.

Finally, since we have noticed that T cell activation pathway seems to be upregulated by triple therapy, we investigated which genes are mostly expressed. For both models we ended up with a list of 20 genes comprising genes such as Cd3d, Ptprc, Cd69, Lat, Lck, Cd2, and Cd3e among others, suggestive of a change in morphology and behavior resulting from exposure to a mitogen, cytokine, chemokine, cellular ligand, or an antigen for which it is specific, as well as APC-to-T cell adhesion (Figure 5A and B).

To further corroborate this hypothesis, we observed which genes are mostly modulated by the therapy. We observed that for 4T1 model 7 genes are differentially expressed (Figure 5C), while for EMT6 model 11 genes (Figure 5D). EnrichR was then used to test for enrichment of these genes for specific signaling pathways, based on annotations from the KEGG database, demonstrating that they modulated pathways involved in antigen procession and presentation for both models (p<0.05) (Figure 5E, F).

The C140, V and CI therapy moves the balance of progenitors exhausted vs terminal exhausted T cells.
We asked what was PD-1 contribution to the treatment. It is widely known that anti-PD-1 favors the selection of progenitors exhausted T cells, which may have beneficial anti-tumoural effects in melanoma patients (33). Progenitors exhausted T cells are defined to be Tim3- (gene name Havcr2), while terminally exhausted T cells, which have poor anti-cancer function, are defined as Tim3+. Therefore, we wondered whether it was the case in our model systems. We checked firstly by scRNAseq whether this treatment increased the frequency of intratumoural tcf1+ stem-like CD8+ T cells (p<0.001) (Figure 6A). Synergy between C140/V and CI might be due, at least in part, to CI selective activity on progenitor exhausted CD8+ TILs, which can respond to anti-PD-1 and were significantly increased in mice treated with chemotherapy and CI when compared to C140 and C140/V treatments (p<0.001) (Figure 6B for both 4T1 and EMT6 models). To further corroborate the scRNAseq data, we performed ex-vivo validation of our data by FACS analysis. Briefly, after tumour injection and treatments, tumours were digested and co-expression of PD-1 and Tim-3 was assessed in CD8+ T cells. Gating strategies are shown in S3A and B. Upon chemotherapy and anti-PD1 treatments, co-expression of PD-1 and Tim3 decreased only in chemotherapy-treated mice. This decrease was more evident when anti-PD1 was present in synergy with both C and V (p<0.05 for EMT6 and p<0.01 for 4T1 model) (Figure 6C).

Consistent with this observation, Tim3+ T cells, previously designated as terminally exhausted cells, were found predominantly in control and chemo-alone groups, whereas the addition of CI to chemotherapy significantly decreased these cells. To further corroborate this hypothesis, we also validated the presence of Entpd1, another marker on terminally exhausted T cell. Consistent with Tim3, also Entpd1 is less expressed in triple treatment, suggesting a selection towards progenitors exhausted T cells in presence of anti-PD-1 (p<0.001) (Figure 7).
Discussion

TNBC is among the most aggressive and lethal types of breast cancer, and currently available therapies have an unsatisfactory impact on patients’ survival. The association of CIs with chemotherapy has shown some encouraging results in randomized clinical trials enrolling TNBC patients, but not all the studies have met their end points and there has been no clear evidence of what should be considered the best chemotherapy backbone to be associated with Cis (6–10). We designed the current study to define, at the preclinical level, what could be considered the most promising combinatorial regimen of chemotherapy plus CI. We investigated different options including P, D, T, V, and C, i.e., the most effective chemotherapy drugs for this disease. Capecitabine (or 5-FU) was not considered as our previous data indicated that C and V were more effective when used in combination with Cis (5).

C is known to elicit potent effects over the immune system including Treg reduction, as well as NK, MDSCs and DC expansion (reviewed in (34)). In fact, the clinical use of C after partially-mismatched hematopoietic stem cell transplant has demonstrated that this drug effectively re-orchestrate also T cells that are pivotal in anti-cancer immunity (3). The use of C as an immunomodulatory drug has been reported both in preclinical models and in clinical trials (11,35-36). In addition to effects on the rewiring of T cells, innate immune cell effector functions are enhanced by C in pathways also involving CD11b+ cells as in the ETM6 model presented here. Present data clearly indicated that intermittent, medium dosage C (C140), in combination with V, was more effective of all other tested combinations. Interestingly, this triple therapy was similarly effective in two models of TNBC that seem to elicit different immunological responses, as in 4T1 tumours the immune cell infiltrate was mainly lymphoid, whereas in ETM6 tumours the infiltrate included in a larger part myeloid (as well as B) cells. In this context, it is noteworthy that myeloid cells, and in particular macrophages, are known to mediate tumour resistance to CIs (37,38). The observed decrease in MHC-II is likely due to the decrease in intratumoral myeloid cells.

To define the mechanisms crucial for C140, V and CIs preclinical activity, we first identified what immune cells populations were needed to control tumour progression. In ETM6-bearing mice with a predominant myeloid and B cell infiltrate (but not in 4T1-bearing mice with a predominant lymphoid infiltrate), neutralization by monoclonal antibodies of CD11b+ myeloid cells, CD19+ B cells and/or CD122+ NK cells slightly reduced the preclinical efficacy of the C140, V and CI therapy, thus suggesting that these immune cell
populations might participate in TNBC control by this triple therapy when the immune cell infiltrate had a relevant myeloid (and B) cell landscape. On the other hand, neutralization studies with monoclonal antibodies against T cells clearly indicated a central, more relevant role for these cells in the preclinical activity of the C140, V and CI therapy. Neutralization of CD3+CD8+ T cells completely abrogated the efficacy of this therapy in 4T1-bearing mice, and neutralization of CD3+CD4+ or CD3+CD8+ T cells completely abrogated the efficacy in ETM6-bearing mice. These partially diverging results underline the need for investigating different in vivo models including infiltrates with myeloid vs lymphoid prevalence.

The central role of T cells in the preclinical activity of C140, V and CI therapy prompted us to investigate the impact of these drugs over different populations of T cells and over their interactions with other players. To this aim, single-cell RNA sequencing provided a robust tool to investigate how seemingly homogenous cell populations may differ in their functional properties when treated with a given drug, and how diverse cell atlases mediate the drug activity (39). Activation of APCs, their processing of tumour antigens and APC interaction with T cells is mandatory for an efficient control of tumour growth by T cells. In fact, several preclinical studies have demonstrated that APCs are major players in the anti-cancer properties of Cis (40–43). As we and others (5,44) have previously shown that V is a potent activator of APCs, we believe that this drug should always be considered and investigated in chemotherapy regimens associated with CIs. The exact mechanism of APC activation by V and the role of B cells are currently investigated in-depth in other companion studies in our laboratory.

Single-cell transcriptome analysis of more than 50,000 intratumoural immune cells indicated that C140, V and CIs induced a unique gene signature suggestive of a behavior associated with exposure to a mitogen, a ligand, or an antigen, as well as APC-to-T cell adhesion. Further analyses in T cells indicated that this triple therapy increased the frequency of intratumoural tcf1+ stem-like CD8+ T cells (45,46). Recent evidence suggested that a successful CI therapy should generate new T cell clones rather than reinvigorate only preexisting tumour-infiltrating lymphocytes (47–49). In this context, it is noteworthy that C140, when compared to P, T, and D, was the drug more efficient in generating new circulating T cells clones (see Graphical Abstract).

We performed a differential gene expression analysis to identify which genes are consistently up- and down-regulated after the triple treatment in T cells. We identified 131 significantly up- and 50 significantly down-regulated genes (Supplementary Table S6). Many of the up-regulated genes are predicted/pseudogenes or ribosomal genes, and pathway
enrichment analysis for these genes did not return any significant results. The pathway enrichment analysis for the down-regulated genes offered more interesting results which deserve to further investigated and validated in future studies.

T cell exhaustion develops in a progressive manner under conditions of prolonged antigen stimulation, leading to a heterogeneous population of exhausted T cells. Recent studies (33,50,51) reported that exhausted T cells exhibit phenotypic and functional heterogeneity, which permits to these cells to be separated in progenitor exhausted and terminally exhausted T populations. The transcription factor T cell factor 1 (Tcf1), encoded by tcf1, is a key regulator of progenitor exhausted T cells. In contrast, terminally exhausted cells express high levels of Tim-3,ENTPD1 and other co-inhibitory receptors (51). Progenitor exhausted T cells (Tcf1+Tim3-ENTPD1-) mediate long-term tumour control and possess stem-like characteristics allowing them to undergo self-renewal and exclusively respond to CI therapy. Terminally exhausted T subsets (Tcf1-Tim3+ENTPD1+) are differentiated from progenitor cells by higher cytotoxicity, but they have reduced long-term survival and are unable to respond to CI.

Our data suggest that the synergy of CIs to chemotherapy is due in part to its selective activity on progenitor exhausted CD8+ TILs, which can respond to CIs and are significantly increased in animals treated with chemo+immunotherapy compared to C140 and C140 plus V treatments alone (Figure 6A-C and Figure 7 for both 4T1 and EMT6). Consistent with this, Tim-3+ T cells, previously designated as terminally exhausted cells, were found predominantly in control and chemo-alone groups, whereas the addition of CIs to chemo leads to a significantly decrease of these cells. The triple combination therapy may alter the balance between the terminally and progenitor exhausted T cells by favoring the latter and improving preclinical efficacy.

Taken together, our observations indicated that the C140, V and CI therapy had a unique activity over the circulating and intratumoural immune cell landscape. These drugs showed a strong preclinical activity in two TNBC models, one associated with a predominant lymphoid intratumoural infiltrated and the other associated with a predominant myeloid/B cell infiltrate. As the dosages of this therapy can be transferred into the clinic with a schedule that seems feasible (11), these findings can be used to design novel clinical trials in this neoplastic disease.
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Author contributions

P.F., S.O., F.B. designed the experiments, analyzed the data and wrote the manuscript. R.H, A.R and L.L. performed all bioinformatic analysis, R.H, A.R, L.L. and P.G.P contributed to manuscript preparation. P.M, C.C supervised the cytometry part and helped to design the experiments.
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**Figure legends**

**Figure 1.** The triple therapy C140+V+anti-PD1 is highly effective in two mouse models of TNBC and relies on T cells. **A)** 4T1 tumour growth curve of mice treated with chemotherapeutic agents, CI or chemotherapeutic agents combined to CI. Dashed line indicates the mastectomy; **B)** EMT6 tumour growth curve of mice treated with chemotherapeutic agents, CI or chemotherapeutic agents combined to CI. The experimental groups are represented in the same way shown in A) Dashed line indicates the mastectomy; **C)** 4T1 quantification of lung metastases in the experimental conditions described in A). Metastases percentage was obtained dividing the area occupied by metastases in the lungs over the whole lung areas. **D)** EMT6 quantification of lung metastases in the experimental conditions described in A). Metastases percentage was obtained dividing the area occupied by metastases in the lungs over the whole lung areas; **E)** 4T1 tumour growth curve of mice treated with triple therapy (C140+V+anti-PD1) and different neutralizing monoclonal antibodies targeting different component of immune cells: CD4+ helper T cells (anti-CD4), CD8+ cytotoxic T cells (anti-CD8), macrophage (anti-CD11b), B cells (anti-CD19), dendritic cells (anti-CD103) and NK (anti-CD122). **F)** EMT6 tumour growth curve of mice treated with identical experimental condition shown in E). (n = 5 per study arm; Shapiro-Wilk Test was applied to define normal distribution. Distribution is normal in graphs A), B), C), F) whilst is not normal in the graph E). Therefore, 2-tails Mann Whitney was applied to E), and T-test of Student was applied to A), B), C), F). Statistic is referred to each condition compared to C140+V+anti-PD1 for graphs A), B), E), F), while is referred to C140+V+anti-PD1 compared to control for the graph E). (*p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001).

**Figure 2.** V induces APC activation, whilst C140 activates T cell clonality. **A)** FACS analysis of CD80 positive dendritic cells (represented in green) over total APC (represented in light-blue) in mice treated with chemotherapeutic agents, CI or chemotherapeutic agents combined to CI injected with 4T1 cells after 3 weeks of treatment. **B)** FACS analysis of CD80 positive dendritic cells as shown in A) in mice injected with EMT6 cells after 3 weeks of treatment. **C)** CD3+CD4+ T cell receptor (TCR) clonality in 4T1 tumours. On x axis 15 common Vbeta chain recombinations are present, while on y axis the fold change increase compared to untreated mice.; **D)** CD3+CD8+ T cell receptor (TCR) clonality in 4T1 tumour, as shown in C); **E)** CD3+ CD4+ T cell receptor (TCR) clonality in EMT6 tumour, as shown in C); **F)** CD3+CD8+ T cell receptor (TCR) clonality in EMT6 tumour as shown in D). (n = 5
per study arm); Shapiro-Wilk Test was applied to define normal distribution. Distribution is normal in graphs A), B), and T-test of Student was applied to A), B). Statistic is referred to each condition compared to each control. (*p < 0.05)

Figure 3. Immune cells landscape in 4T1 and EMT6 tumours by scRNA-seq analysis.
A) Immune cell landscape analysed by scRNA-seq in 4T1 tumour in the different experimental conditions. The top panel shows the Uniform Manifold Approximation and Projection (UMAP) with cells coloured by the immune cell type they were assigned to, whilst the bar graph in the lower panel represents the different immune cell abundance within each treatment. B) Immune cell landscape analysed by scRNA-seq in EMT6 tumour in the different experimental conditions. The top panel shows the uniform manifold approximation and projection (UMAP) with cells coloured by the immune cell type they were assigned to, whilst the bar graph in the lower panel represents the different immune cell abundance within each treatment.

Figure 4. Triple therapy promotes T cell activation and proliferation.
A) Differences in pathway activities scored by GSVA between control and triple treatment (C140+V+anti-PD1), and B) between triple and C140+V treatments for T cell as assigned by singleR. t scores from a linear model are shown with the threshold set to 3 (grey pathways) Left panel for the 4T1 T cells, and right panel for the EMT6 T cells. C) T cell activity estimated through module score and calculated for genes Cd27, Cd28, Cd69, Cd25 and Interferon gamma in 4T1 (left) and EMT6 (right) tumour immune cells for the different experimental conditions. D) T cell activity showed as in C) but presented as expression, red line refers to median value.

Figure 5. Triple therapy regulates different genes involved in antigen presenting and presentation.
A) 20 Top expressed genes in 4T1 model by triple therapy in the GO term “T cell activation”. B) 20 Top expressed genes in 4T1 model by triple therapy in the GO term “T cell activation”. C) Expression of differentially expressed genes (DEGs) from GO term “T cell activation” in triple treatment (C140+V+anti-PD1) in 4T1 cells compared to cells from all other treatments combined. D) Expression of differentially expressed genes (DEGs) from GO term “T cell activation” in triple treatment (C140+V+anti-PD1) in EMT6 cells compared to cells from all other treatments combined. E) Enrichr results from “KEGG mouse 2019” database for
DEGs identified for triple treatment in 4T1 cells show enrichment in “Antigen processing and presentation”. Taken from Ernichr site: https://amp.pharm.mssm.edu/Enrichr/ F) Enrichr results from “KEGG mouse 2019” database for DEGs identified for triple treatment in 4T1 cells show enrichment in “Antigen processing and presentation”. Taken from Ernichr site: https://amp.pharm.mssm.edu/Enrichr/

Figure 6. Triple therapy selects terminally exhausted T cells.
A) Tcf1 expression in 4T1 (left) and in EMT6 (right) tumour immune cells split by treatment shown as violin plot. B) Expression of Tim3 (Havcr2) in 4T1 (left) and in EMT6 (right) tumour immune cells split by experimental condition shown as violin plot. C) Intratumoral percentage of PD-1+TIM3+ CD3+CD8+ T cells is shown in the bar graph in 4T1 (on the left) and in EMT6 model (on the right). Cells were plotted calculating the percentage of positive cells over the lymphocytes. (n = 5 per study arm); Shapiro-Wilk Test was applied to define normal distribution. Distribution is normal in both graphs and T-test of Student was applied. Statistic is referred to each condition compared to control. (*p < 0.05, **p < 0.01).

Figure 7. PD-1 in combination with chemotherapy favours progenitors exhausted T cells
Expression of Entpd1 (Entpd1) in 4T1 (left) and in EMT6 (right) tumour immune cells split by experimental condition shown as violin plot. 2-tails Mann Whitney was applied (*p < 0.05, **p < 0.01, ***p < 0.001).
Figure 1

A. 4T1

B. EMT6

C. 4T1

D. EMT6

E. 4T1

F. EMT6

Tumor volume (mm$^3$) vs. Days post tumor injection for 4T1 and EMT6 cell lines. The graphs show the tumor volume measured over time for different experimental conditions, with control groups and various treatments indicated.

% of metastasis/lung surface vs. Days post tumor injection for 4T1 and EMT6 cell lines. The bars represent the percentage of metastasis relative to the lung surface area, with different conditions indicated.

Tumor volume (mm$^3$) vs. Days post tumor injection for 4T1 and EMT6 cell lines, with the effects of different treatments on tumor growth shown.

Note: The graphs illustrate the impact of various treatments on tumor growth and metastasis, with statistical significance indicated by asterisks (*, **, ***).
Figure 2

A. Bar graph showing the percentage of CD80+ cells over total APCs for 4T1 cells in different conditions: Control, aPD1, D+aPD1, T+aPD1, P+aPD1, C140, C140+aPD1, C140+V, C140+V+aPD1. The x-axis represents different conditions, and the y-axis shows the percentage of CD80+ cells.

B. Bar graph similar to A for EMT6 cells.

C. Fold change graph for 4T1 CD4+ cells showing the expression of vβ chains under different conditions.

D. Fold change graph for 4T1 CD8+ cells showing the expression of vβ chains.

E. Fold change graph for EMT6 CD4+ cells.

F. Fold change graph for EMT6 CD8+ cells.
Figure 3

A

4T1

B

EMT6

UMAP 2

UMAP 1

Percentage (%)

Percentage (%)

control αPD1 D+αPD1 T+αPD1 P+αPD1 Cy140 Cy140+αPD1 Cy140+V+αPD1

B cells ILC Monocytes NK cells Others

Dendritic Cells Macrophages Neutrophils NKT T cells CD4 Helper

T cells CD4 Mem T cells CD8 Citotoxic T cells CD8 Naive T cells CD8 Mem Tgd

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Figure 6

A  4T1

Expression level of Tcf1

B  4T1

Expression level of Havcr2

C  4T1

%CD3+CD8+PD-1+Tim3+/Lymphocytes

EMT6

Expression level of Tcf1

Expression level of Havcr2

%CD3+CD8+PD-1+Tim3+/Lymphocytes
Cyclophosphamide and vinorelbine activate stem-like CD8+ T cells and improve anti-PD-1 efficacy in triple-negative breast cancer

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