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Original Research

Type of chemotherapy has substantial effects on the immune system in ovarian cancer

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ARTICLE INFO

Keywords:

Ovarian cancer

Chemotherapy

MDSC

Carboplatin-paclitaxel

Immunosuppression

ABSTRACT

Chemotherapy induces a variety of immunological changes. Studying these effects can reveal opportunities for successful combining chemotherapy and immunotherapy. Immuno-chemotherapeutic combinations in ovarian cancer are currently not generating the anticipated positive effects. To date, only scattered and inconsistent information is available about the immune-induced changes by chemotherapy in ovarian cancer. In this study, we compared six common chemotherapeutics used in ovarian cancer patients (carboplatin, paclitaxel, pegylated liposomal doxorubicin, gemcitabine, carboplatin-paclitaxel and carboplatin-gemcitabine) and studied their effects on the immune system in an ovarian cancer mouse model. Mice received a single chemotherapy or vehicle injection 21 days after tumor inoculation with ID8-fluc cells. One week after therapy administration, we collected peritoneal washings for flow cytometry, serum for cytokine analysis with cytometric bead array and tumor biopsies for immunohistochemistry. Carboplatin-paclitaxel showed the most favorable profile with a decrease in immunosuppressive cells in the peritoneal cavity and an increase of interferon-gamma in serum. In contrast, carboplatin-gemcitabine seemed to promote a hostile immune environment with an increase in regulatory T-cells in tumor tissue and an increase of macrophage-inflammatory-protein-1-beta in the serum.

Introduction

Ovarian cancer (OC) is a highly aggressive cancer responsible for the death of 28 253 woman in Europe each year (Globocan). Standard-of-care consists of tumor debulking surgery and platin-based chemotherapy. Despite major advances in immunotherapy, ovarian cancer patients have disappointing response rates to immunotherapies in monotherapy and even in combinations [1–3]. Despite this failure, chemotherapy is still deemed as a useful ally to boost the efficacy of immunotherapies because of the immune-manipulating properties of chemotherapeutics.

Chemotherapy can induce a wide range of effects on multiple levels that are already extensively investigated and reviewed elsewhere [4,5]. Generally, we can summarize the effects into three main categories. A first and major effect of chemotherapy is the depletion of fast dividing cells, including the bone marrow progenitor cells and the ac-

tive, proliferating immune cells at the tumor site. This can result in both immune suppression by inducing lymphopenia as well as immune activation since the immune suppressive network of both tumor cells and (immune suppressive) immune cells can be disrupted. A second effect of chemotherapy is the increased induction of (neo)antigens and the release of antigenicity during cell death. However, for ovarian cancer, O'Donnell et al. showed only a marginal effect of chemotherapy on increase of neoantigen expression of only 5% from the total of 78% more expressed neoantigens, indicating that other processes (e.g. mutagenesis) play a larger role in neoantigen formation [6]. A third effect is the induction of immunogenic-cell death, an increased availability of appropriate immunostimulatory signals to stimulate the anti-tumor immunity response [7].

However, the immunological changes induced by chemotherapy are not as straightforward as they may seem. The first difficulty is that there

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<https://doi.org/10.1016/j.tranon.2021.101076>

Received 18 December 2020; Received in revised form 15 February 2021; Accepted 10 March 2021

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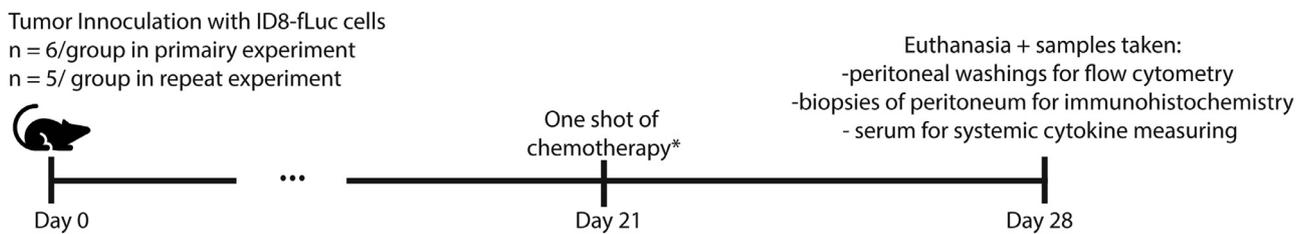


Fig. 1. Overview of set-up of the experiments. *Group 1: carboplatin + gemcitabine, group 2: carboplatin + paclitaxel, group 3: Pegylated Liposomal Doxorubicin group 4: carboplatin, group 5: paclitaxel, group 6: gemcitabine.

are different immune-effects when using different dosages of chemotherapeutics [8]. In a HM-1 ovarian cancer mouse model, it was shown that a dose dense chemotherapy (DD) schedule, compared to the maximum tolerated dose regimen, could preserve CD8⁺, CD4⁺ and CD11b⁺ cells, increase F4/80⁺ cell recruitment into the tumor and reduce the numbers of myeloid derived suppressor cells (MDSC) [9]. The immune related effect of DD schedule could also be correlated with an improved control of tumor growth. In patients with advanced or recurrent OC, the DD schedule was well-tolerated and had a better outcome, while maintaining a constant level of leucocyte numbers [10,11]. The fact that chemotherapy will not only modulate the immune system but also its direct environment, makes the situation more complex. Moreover, the effect on the immune cells is also dual, since it can be an effect on the number of cells present but also an effect on the activity of the immune cell. Moschella et al. highlighted this in their study where they profiled genetic responses of peripheral blood mononuclear cells (PBMC) of OC tumor-bearing mice treated with cyclophosphamide and found in the bone marrow PBMC alone 1123 differentially regulated genes, added with another 1083 and 868 genes in respectively blood and spleen PBMC [12]. A last problem lies in the fact that the effects of one chemotherapeutic agent in one type of cancer cannot be generalized to other cancer types. A comparison of cisplatin or carboplatin treated ovarian and cervical cancer cell lines by Dijkgraaf et al. showed that the ovarian cancer cell lines COV413B, CAOV3, and cervical cancer cell lines HELA, C83, CSCC7 upregulated their interleukin-6 (IL-6) expression upon treatment and subsequently skewed monocytes towards an M2-like phenotype. In contrast, the same treatment on the other ovarian cancer cell lines (SKOV3, OVCAR3, A2780) or cervical cancer cell lines (CCSCC1, CAKI) did not increase IL-6 production or the skewing of monocyte differentiation [13]. Lastly, we and others have shown that not only attention should be given to the type of therapies combined, but also the order and timing, is crucial [11,14]. These remarks exemplify that a deeper knowledge of the mechanisms behind chemotherapy driven immune changes is required. In ovarian cancer, this information is scattered and set-up of experiments are too heterogenous to draw conclusions (for review, see supplementary Table 1).

The goal of this research was to investigate and compare the immune modulating effects of the most commonly used chemotherapies in ovarian cancer patients. To this end, we used the ID8-fLuc ovarian cancer mouse model [15]. Like this, we hope to identify positive effects that can be used when designing immunotherapy-chemotherapy combinations.

Material and methods

Ovarian cancer mouse model

Six to eight-week-old, female C57BL/6 mice (Envigo, Horst, The Netherlands) were intraperitoneally (ip) injected with 5×10^6 ID8-fLuc cells and randomly labelled. Approval of the ethical committee was obtained (P075/2014) and ethical standards (NIH guidelines for the Care and Use of Laboratory Animals) as well as the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines were strictly fol-

lowed [16,17]. Sample size was determined via a statistical power analysis, where five mice per treatment group had a power of 0.7320 to detect differences between groups. Mice were co-housed per five mice in individually ventilated cages (IVC) at the Specific-pathogen-free (SPF) facilities of the KU Leuven. An overview of the design of the experiment is given in Fig. 1.

Chemotherapeutic treatments in mice

Different treatments was randomly allocated to the mice. All drugs were administered ip in the right flank of the mouse abdomen, three weeks after tumor inoculation. Controls were injected with Dulbecco's phosphate-buffered saline (DPBS, Thermofisher). If combinations of chemotherapies were required (for example carboplatin-paclitaxel), two separate administrations of each chemotherapeutic were given at the same moment. Carboplatin (Hospira, ONCO-TAIN) was dissolved in glucose 5% and administered ip in a dose of 100 mg/kg. Paclitaxel (AB, Aurorobindo Pharma B.V) was diluted in NaCl 0,9% and administered ip in a dose of 10 mg/kg. Gemcitabine (Hospira Benelux BVBA – BE390476) was diluted in NaCl 0,9% and administered ip in a dose of 50 mg/kg in monotherapy and in a dose of 120 mg/kg when combined with carboplatin. Pegylated liposomal doxorubicin (PLD, Caelyx®, Janssens Cilag International NV) was administered ip in a dose of 6 mg/kg. Animals which became severely ill due to chemotherapy toxicities (e.g. weight loss, diarrhea and cachexia) were sacrificed prematurely and were excluded from data analysis.

Sampling of blood, peritoneal fluid and tumor tissue for immune monitoring

Immunological changes were evaluated one week after chemotherapy administration. This time point was chosen to study delayed immune effects, rather than the immediate effect as well as to increase the translational relevance of our experiments in correspondence to certain combinatorial immunotherapy trials where chemotherapy is started prior to immunotherapy (e.g. DUO-O (NCT03737643)). Mice were anesthetized with pentobarbital (200 mg/ml, Dolethal, Vetoquinol) prior to blood collection. Whole blood was centrifuged for ten minutes at 5000 rcf to obtain serum. Serum samples were aliquoted and stored at -80°C until cytokine quantification with cytometric bead assay. Next, mice were euthanized and a peritoneal lavage was performed using 10 mL DPBS. The peritoneal washings were centrifuged for five minutes at 500 rcf. The cell pellet was resuspended and used in fluorescent activating cell sorting read out. Biopsies of the peritoneum for immunohistochemical stainings were taken immediately after euthanasia of the mice and fixed by 4% paraformaldehyde.

Fluorescent-activated cell sorting (FACS)

The cell pellet was dissolved in DPBS and stained with eFluor780 fixable viability dye (Affymetrix Inc. San Diego, Ca, USA) in order to exclude dead cells. Next, surface markers were stained for myeloid cell markers and T-cell markers with monoclonal antibodies as described in supplementary Table 2. In addition, intracellular markers were stained.

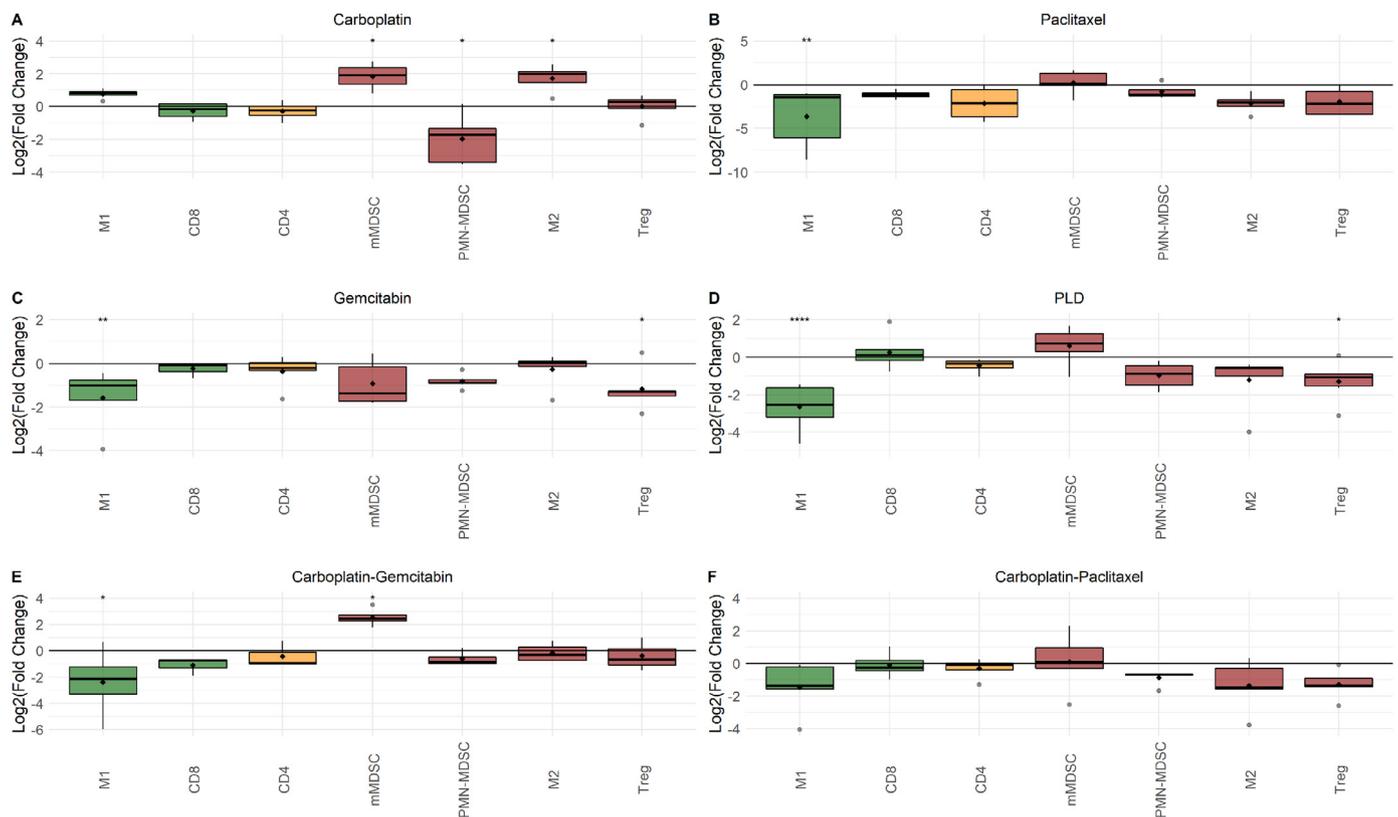


Fig. 2. Fold change (transformed with base log₂) of immune profile one week after chemotherapy administration versus tumor bearing non-treated mice. A: Carboplatin induces an increase in mMDSC (mean = 1,8, **p*<0.05), a decrease in PMN-MDSC (mean = -1,9, ***p*<0.01) and an increase in M2 (mean = 1,7, **p*<0.05). B: Paclitaxel induces a mean 3,65-fold decrease in M1 (***p*<0.01). C: Gemcitabine induces a mean 1,57 decrease in M1 (***p*<0.01) and a mean 1,17 decrease in Treg (**p*<0.05). D: PLD induces a 2647-fold decrease in M1 (*****p*<0,0001) and a 1,29-fold decrease in Treg (**p*<0.05). E: Carboplatin-Gemcitabine shows a significant 2,54-fold increase in mMDSC (**p*<0.05) and a 2,4-fold decrease in M1 (**p*<0.05). F: Carboplatin-Paclitaxel shows no significant differences in immune profile. The fold change is calculated by dividing the amount of immune cells (as a percentage of their parent-population) in mice one week after chemotherapy (individual values) by the amount of immune cells in control mice (mean of the group). PLD = Pegylated liposomal doxorubicin. All tests are one-way ANOVA. Gray dots represent outliers, black squares in bar plot represent mean, line represents median. (*N* = 6 per group in first experiment, *N* = 5 per group in repeat experiment).

For the myeloid panel, cells were permeabilized using Leucoperm (Bio-Rad Laboratories Inc., Kidlington, UK) in accordance to manufacturers' protocol and in addition stained for the intracellular marker CD206. Fluorescence Minus One technique was used to control for the gating of the marker CD206. For the T cell panel, cells were permeabilized using eBioscience Foxp3 / Transcription Factor Staining Buffer Set (ThermoFisher Scientific, Waltham, Massachusetts, USA) and were stained for the intracellular marker Foxp3. All antibodies used were titrated for optimal concentration. Acquisition was performed using FACS DIVA software on the BD Canto II (BD bioscience). FlowJo Single Cell Analysis software (TreeStar, Inc., Ashland, OR, USA) was used to analyze the data.

Immunohistochemistry

Immunohistochemistry single marker stains were performed for CD8⁺ cytotoxic T-lymphocytes, FoxP3⁺ regulatory T-lymphocytes, Ly6C⁺ myeloid derived suppressor cells and F4/80⁺ macrophages as described earlier [18]. Rat anti-mouse CD8a clone 4SM15 (e-Bioscience 14-0808-82) was used in a 1:100 dilution. Rat anti-mouse Foxp3 (e-Bioscience 14-5773-82) was used in a 1:100 dilution. Monoclonal Rabbit anti-mouse F4/80 (D2S9Rxp) (BioKé, Cell Signaling 70,076 s) was used in a 1:250 dilution. Rat anti-mouse Ly6C, clone ER-MP20 (ThermoFisherScientific MA1_8189) was used in a 1:200 dilution. Microscopic images were digitalized using the Zeiss Axio Slide Scanner using a x20 objective and ZEN2 software (Zeiss). QuPath was used for digital, manual analysis [19]. Per slide, four different region of interest (ROI) containing tumor tissue were selected. Positive cells were counted per

ROI and a ratio was made of positively counted cells divided by the ROI in μm^2 .

Cytometric bead assay

Serum samples were analyzed for cytokine expression. The following cytokines were measured using the cytometric bead assay technique (BD Biosciences, San Jose, CA, USA): interleukin (IL-) 1 β , IL-6, IL-10, IL-12p70, monocyte chemoattractant protein-1 (MCP-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein 1 α (MIP-1 α), MIP-1 β , interferon gamma (IFN γ), tumor necrosis factor (TNF). Undiluted samples were analyzed according to manufacturers' protocol using flex sets. Acquisition was performed on the BD Fortessa (BD Biosciences, San Jose, CA, USA). Cytokine analysis was performed using FCAP Array Software v3.0 (BD Biosciences, San Jose, CA, USA). In some treatment groups the concentration of the measured cytokines was too low to detect following the CBA-standard curve and manufactures threshold levels. Values below this threshold were excluded for statistical analysis.

Results

Gemcitabine, paclitaxel and carboplatin-paclitaxel reduce immunosuppression in ascites

After administration of carboplatin, paclitaxel, gemcitabine and paclitaxel as a monotherapy, we observed that with carboplatin the

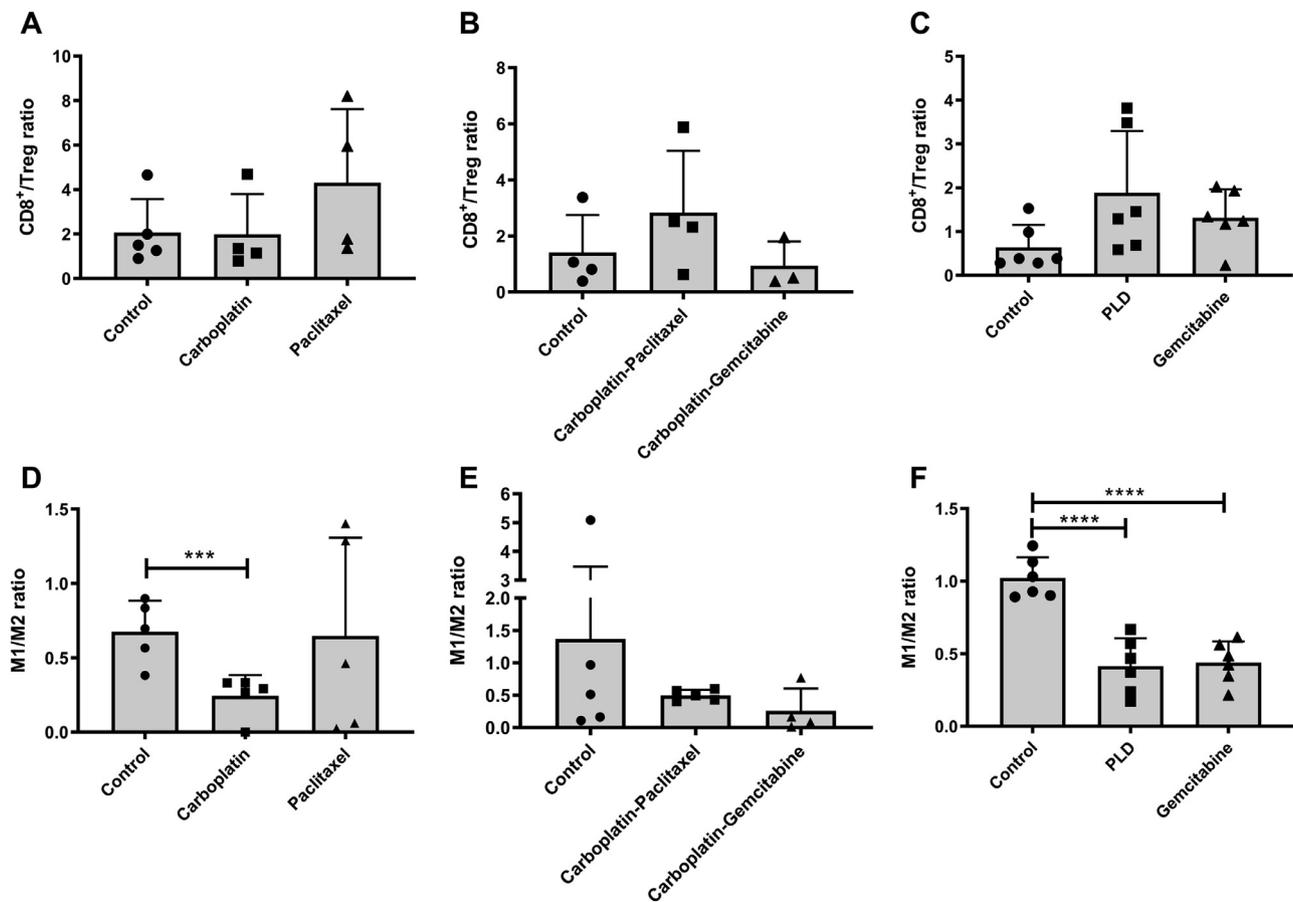


Fig. 3. CD8⁺/Treg and M1/M2 ratios to evaluate immune profile after chemotherapy administration evaluated by flow cytometry. A-C: no significant differences when the CD8⁺/Treg ratio is made for all the different chemotherapies. D: Carboplatin shows a significant lower M1/M2 ratio ($p = 0,0079$, Mann-Whitney U test). E: no significant differences in M1/M2 ratios for the chemotherapeutic combinations of Carboplatin-Paclitaxel and Carboplatin-Gemcitabine. F: Significant lower M1/M2 ratio when PLD is compared to control-treated mice ($p < 0,0001$, unpaired student *t*-test) and Gemcitabine is compared to control treated mice ($p < 0,0001$, unpaired student *t*-test). PLD = Pegylated liposomal doxorubicin. Ratio's were calculated using number of immune cells as percentage of parent-population. ($N = 6$ per group in first experiment, $N = 5$ per group in repeat experiment (results of repeat experiment shown)).

amount of immune suppressive monocytic MDSC (mMDSC) increased 3,5 times. In contrast, the polymorphonuclear MDSC (PMN-MDSC) showed an almost equal decrease of 3,7x. In addition, carboplatin monotherapy caused 3,2x more of type 2 pro-tumoral macrophages (M2) present in the peritoneal cavity compared to control-treated tumor bearing mice (Fig 2A). Paclitaxel monotherapy induced a 12,6x decrease in type I anti-tumoral macrophages (M1), displaying an inflamed phenotype, compared to control-treated tumor bearing mice (Fig 2B). Upon treatment with gemcitabine, a decrease of approximately 3x fewer M1 and 2,3x fewer Treg were detected (Fig 2C). PLD induced a decrease of 5,9x less M1 and 2,4x less Treg (Fig 2D). The combination of carboplatin-gemcitabine treatment resulted in on average 5,7x more mMDSC and 5,2x less M1 (Fig 2E). The combination of carboplatin-paclitaxel did not induce any significant changes in the immune composition (Fig 2F). In addition, we observed that carboplatin, PLD and gemcitabine in monotherapy resulted in a significant decrease of the M1/M2 ratio (Fig. 3D-F). None of the chemotherapeutic agents were able to induce a relevant change in the CD8/Treg ratio (Fig. 3A-C).

Carboplatin-gemcitabine increases regulatory T cells in tumor tissue

Apart from carboplatin-gemcitabine that induced a significant increase in FOXP3⁺ cells, we recorded no statistically significant changes in the intratumoral immune composition, after administration of any chemotherapy regimens (Fig. 4).

Carboplatin-gemcitabine increases the concentration of macrophage inflammatory protein 1 beta

Serum cytokines were measured in a total of 84 mice over two independent experiments. However, most cytokine concentrations remained out of detectable range (OOR) (Fig 5A). The cytokines MIP-1a, IL-1B, IL-10, GM-CSF and IL-12p70 were not detectable in over half of the samples and therefore excluded from further statistical analysis. The only cytokine measured repeatedly was MIP-1b, which showed a significant increase in mice treated with carboplatin-gemcitabine (Fig 5B). Cytokines TNF, MCP-1, IL-6 and IFN γ could be detected in the majority of mice treated with carboplatin, paclitaxel, carboplatin-paclitaxel and carboplatin-gemcitabine. Overall, carboplatin-gemcitabine treatment induced a major increase in all studied serum cytokines, whereas paclitaxel barely induced any changes. Carboplatin in monotherapy induced a significant increase IFN γ .

Discussion

To the best of our knowledge, information on the effect of chemotherapy on the immune system in ovarian cancer bearing mice was only limitedly available. In our study, we evaluated the effects at the tumor tissue level, in ascites and in blood. Our results demonstrate favorable changes of the immune system by carboplatin-paclitaxel (decrease of immunosuppressive cells in ascites and increase of IFN γ in serum) and a worsening of the immune microenvironment by carboplatin-

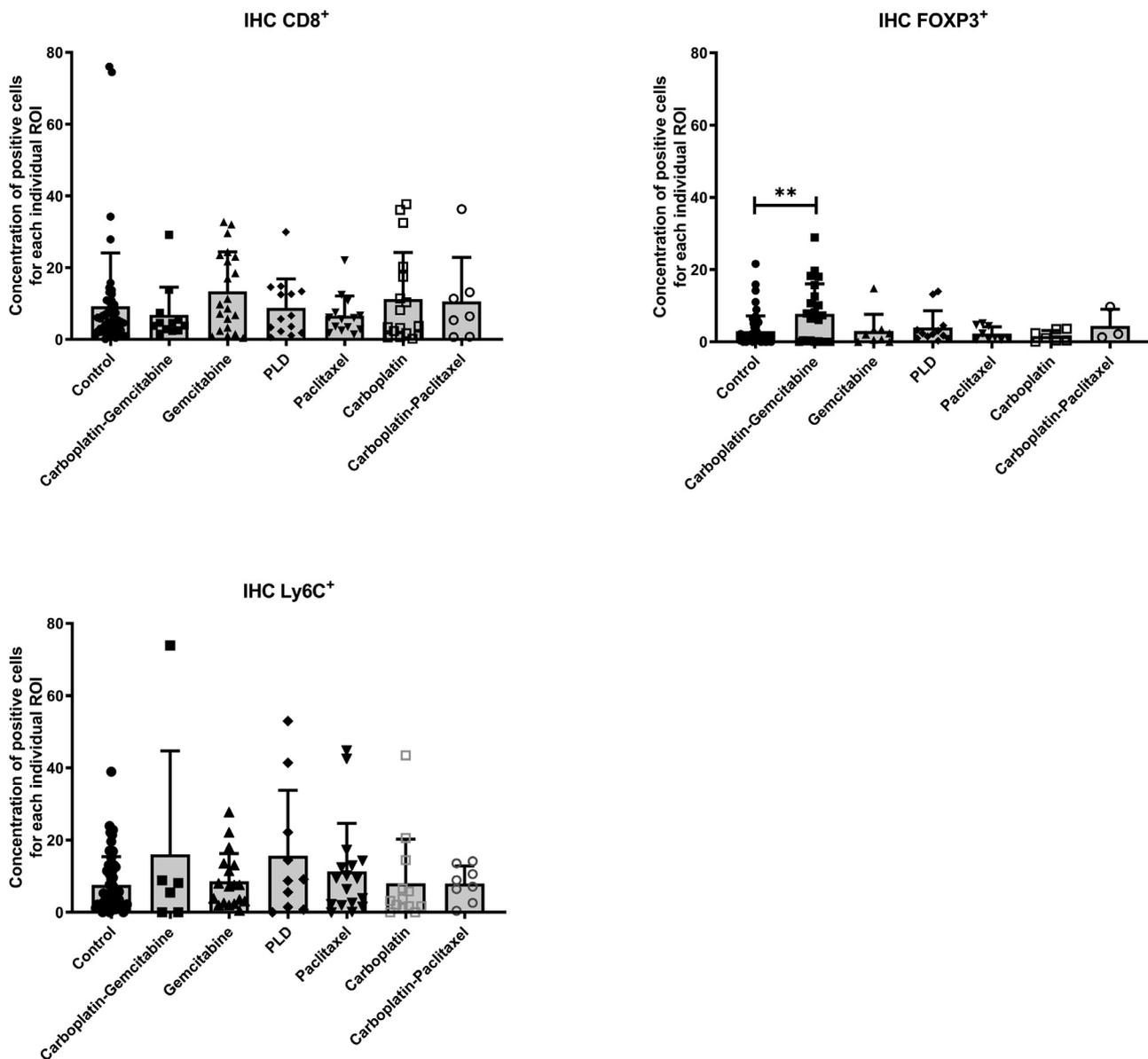


Fig. 4. Immunohistochemical analysis of peritoneal biopsies. Carboplatin-Gemcitabine treated mice show an increase in intratumoral FoxP3⁺ cells (p -adj=0,0014, one-way ANOVA). No significant intratumoral differences could be noted in CD8⁺ and Ly6C⁺ cells. PLD = pegylated liposomal doxorubicin. ($N = 6$ per group in first experiment, $N = 5$ per group in repeat experiment, four different regions of interest were measured in each individual sample (results of initial experiment shown)).

gemcitabine (increase in Treg in tumor tissue and increase in MIP1 β in serum) (Fig. 6). The effects of chemotherapy in monotherapy are either absent or only present at one read out level and therefore represent most likely a less strong influence on the immune system. These findings are crucial in our understanding of the immune system in ovarian cancer and for the design of preclinical experiments, combining chemotherapy and immunotherapy, that should precede clinical trials.

To date, no immunotherapeutic strategies have been implemented successfully in the routine treatment of ovarian cancer, despite prove that ovarian cancer is indeed an immune responsive malignancy. A first milestone was set by Zhang et al. who showed that T-cell infiltration correlated with an improved 5-year survival of 73.9% versus only 11.9% for patients without. Both the KEYNOTE-100 (Phase II in advanced recurrent ovarian cancer) and the JAVELIN Solid tumor (ovarian cohort) trial using pembrolizumab and avelumab were therefore promising candidates, but ended up with disappointing results, yielding overall response rates of less than 10% in monotherapy [20,21]. Part of this failure could be attributed to the high levels of immune suppression present

in ovarian cancer, represented by Treg, MDSC and M2. Indeed, in ovarian cancer, this influx of innate immune suppressive cells has been correlated with worsened survival [18,22]. Moreover, these cells are not the subject of current immunotherapeutic strategies. Therefore, part of the solution could lie in a strategic combination of chemotherapy, immunotherapy and targeted therapy combinations to overcome immunosuppression. Knowledge about the immune-effects of chemotherapy is crucial in designing these combinations. Our data show that carboplatin-gemcitabine seems to create an increase in immune suppression as it endorses the innate immune suppressive cells mMDSC and decreases the numbers of M1. In addition, carbo-gemcitabine increases the serum protein levels of MIP-1 β . Giuntoli et al. found elevated levels of MIP-1 β in both ascites and serum samples of patients at the time of primary cytoreductive surgery [23]. Taken this together, carboplatin-gemcitabine does not seem to be the chemotherapeutic of choice to be used in combination strategies. For PLD chemotherapy, we found an induction of a low M1/M2 ratio. Clinical data in ovarian cancer has demonstrated that a low M1/M2 ratio was associated with a worse prognosis [24], making

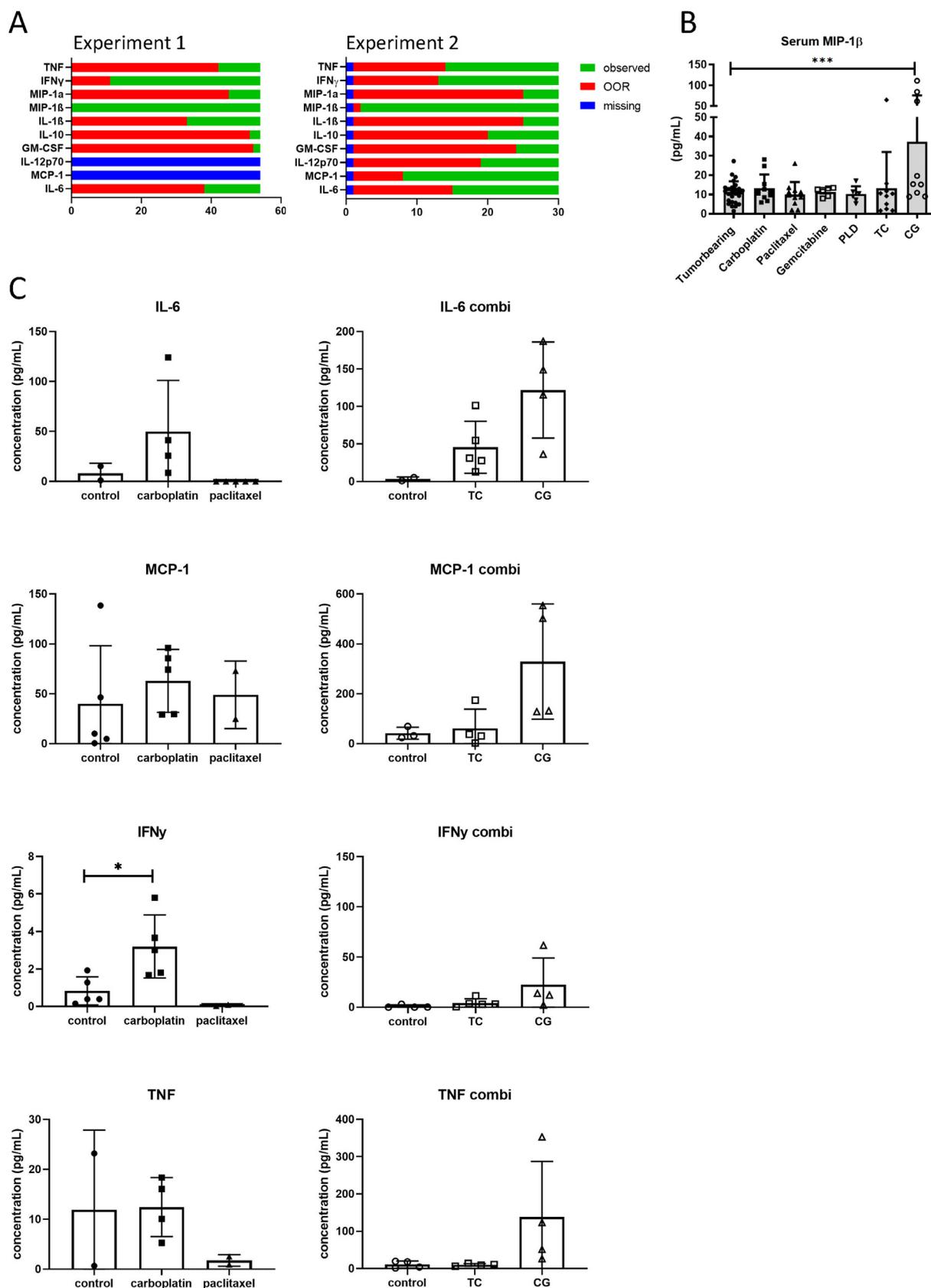


Fig. 5. Serum cytokines measured one week after chemotherapy administration. A: Cytokines MIP-1 β (CCL4), IL-10, IFN- γ , MCP-1 (CCL2), IL-6, TNF- α , IL-12p70, GM-CSF, MIP-1 α (CCL3) were measured with CBA in a total of 84 mice in two separate experiments. Most cytokines remained out of detectable range (OOR). B: MIP-1 β was measurable across all treatment groups and showed a significant increase (** $p < 0.0002$) for CG compared to control. C: TNF, MCP-1, IL-6 and IFN γ were measurable in the treatment groups carboplatin, paclitaxel, TC and CG. Carboplatin showed a significant increase in IFN- γ ($p < 0.05$). ($N = 6$ per group in first experiment, $N = 5$ per group in repeat experiment (results of both experiments combined are shown)). P-values were computed by comparing treated cytokine values to control group, via one-way ANOVA.

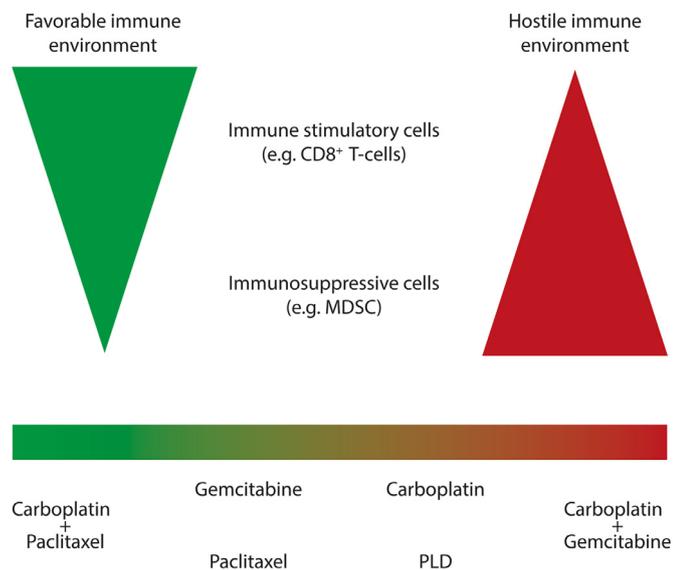


Fig. 6. Outline displaying the compared chemotherapeutics and their overall immune effect.

these effects also undesirable. Gemcitabine in monotherapy decreased the number of Treg, but also exhibited a lower M1/M2 ratio. This unfavorable lower M1/M2 ratio, could impede the positive effects of lower Treg numbers, making also this chemotherapeutic a lesser choice in combination strategies. Also carboplatin in monotherapy showed the same unfavorable lower M1/M2 ratio while in serum we detected increased $IFN\gamma$ levels. Although $IFN\gamma$ is generally regarded as a pro-inflammatory cytokine, it has been shown that $IFN\gamma$ upregulates the expression of PD-L1 in ovarian cancer cells and thereby can inhibit an antitumoral immune response [23]. An additional important observation is that the immune modulating effect of dual chemotherapies (carboplatin-paclitaxel or carboplatin-gemcitabine) is not a summation of these effects of the chemotherapies in monotherapy.

In contrast to literature, we do not see a toxicity towards MDSC under gemcitabine treatment in mice [25–27]. One explanation for this discrepancy is that the mouse models used in these studies were subcutaneous tumor models of breast cancer and pancreatic cancer, where they studied mostly the presence of MDSC at the level of the spleen, which is in contrast to our work, based on an orthotopic mouse model and the evaluation of MDSC in ascites. However, it is also true that not all tumors respond equally and this highlights once more the important differences between the immune biology of the different tumors. In contrast to Peng et al., we did not find an increase in $CD4^+$ and $CD8^+$ cells after paclitaxel treatment. In general, we did not see many immune changes on an intratumoral level. Only the carboplatin-gemcitabine treatment could induce an increase in $FOXP3^+$. A possible explanation for these contrasting results is that both the paclitaxel dose and the time point of analyses differs [28]. Another explanation can be found in the fact that the immune composition is different between different metastatic biopsies in ovarian cancer, as is demonstrated in three recent papers [29–31]. Therefore, we should be cautious with interpreting and extrapolating results from a single and relatively small tumor biopsy, as it may not be representative for the whole tumor or the general immune condition of the patient.

We would like to acknowledge some important limitations to this study. We recognize the absence of data on the activation status and functional capacities of the immune cells, as our study focused on exploring shifts in numbers of immune cells caused by the different chemotherapies, and that the immune system was only evaluated at one time point. This time point was chosen to see the delayed immune effects of the chemotherapy, since evidence already exists that chemotherapy can directly stimulate effector functions of several immune cells [32]. How-

ever, more time points should be investigated when looking for the optimal therapeutic windows. Another limitation is that we investigated the effect of only one chemotherapy administration, while in a clinical setting, patients will receive multiple cycles of chemotherapy.

We believe our findings are crucial to design combinatorial immunotherapy trials. At present, it is not known how to combine chemotherapy and immunotherapy. Preclinical evidence is lacking and clinical immunotherapy studies have been stopped prematurely or were negative [20,21,33]. Besides the fact that immune monitoring in clinical immunotherapy trials will be crucial to understand how the immune biology of patients is altered [34], it will be necessary to preclinically test combination regimens. To successfully combine therapies, the knowledge of the immune modulating effects of chemotherapy is crucial.

In conclusion, we have provided information on the immunological effects of chemotherapy in an ovarian cancer mouse model. Based on these results, carboplatin-paclitaxel induced a superior immune profile to that of paclitaxel, carboplatin, gemcitabine or carboplatin-gemcitabine and therefore could be considered for future combined chemotherapy-immunotherapy treatment regimens. We are thus hopeful and patiently awaiting results of some of the newer trials (e.g. DUO-O (NCT03737643), FIRST (NCT03602859) or ATHENA (NCT03522246)), who are studying in a first-line setting the combination of checkpoint inhibitors with carboplatin-paclitaxel.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Formating of funding sources

This work was supported by Kom Op Tegen Kanker (Stand up to Cancer), the Flemish cancer society (2016/10728/2603 and 2019/11955/1 to AC and 11758 to AVK); the Olivia Fund (2017/LUF/00135 to AC); Amgen Chair for Therapeutic Advances in Ovarian Cancer (2017/LUF/00069 to IV)

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101076.

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