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IMMUNOMODULATORY EFFECTS OF MONONUCLEAR 5,6-EPOXY-5,6-DIHYDRO-1,10-PHENANTHROLINE PLATINUM(II) COMPLEX

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ABSTRACT. The newly developed mononuclear 5,6-epoxy-5,6-dihydro-1,10-phenanthroline platinum(II) complex revealed notable antitumor effects *in vitro* and *in vivo*. In this study, the effects of this platinum(II) complex on the immune response were assessed. Peritoneal macrophages and splenocytes obtained from mice were treated with lipopolysaccharide (LPS)/Concanavalin A (ConA) along with platinum(II) complex and measurement of cytokine concentrations and immunophenotyping was performed. Our findings indicate that the platinum(II) complex exhibits significant immunomodulatory effects on peritoneal macrophages and splenocytes.

Keywords: platinum(II) complex, macrophages, splenocytes, cytokines.

INTRODUCTION

Malignancies are a significant health concern, and they represent major and leading causes of death worldwide (ADEBOWALE *et al.*, 2015). Consequently, there is growing interest in developing new metal-based complexes that can overcome the limitations of conventional therapy, while simultaneously retaining their therapeutic

efficiency (MUHAMMAD *et al.*, 2014; SIMPSON *et al.*, 2019). Platinum-based drugs are one of the most commonly used chemotherapeutics (ARSENIJEVIC *et al.*, 2017). They are highly effective against testicular, ovarian, bladder, head and neck, esophageal, small-cell and non-small-cell lung cancers as well as lymphoma and osteosarcoma (ADELSTEIN *et al.*, 1989; ABRATT *et al.*, 1992; ABALI *et al.*, 2008; ABEDINI *et al.*, 2008; ABDEL KARIM *et al.*, 2015; ABE *et al.*, 2019; AGRAWAL *et al.*, 2019; ABDELGWAD *et al.*, 2022; A BUENO *et al.*, 2023). However, severe side effects and drug resistance present great limitations in their efficiency. Side effects include nephrotoxicity, hepatotoxicity, neurotoxicity, ototoxicity, myelosuppression, leucopenia, emesis, alopecia, and immunosuppression (ADEBOWALE *et al.*, 2015; FRONIK *et al.*, 2021). There are numerous different platinum complexes under investigation currently, while in clinical practice only cisplatin, oxaliplatin, and carboplatin are used (DIMITRIJEVIĆ STOJANOVIĆ *et al.*, 2022). Our previous study suggests that novel mononuclear 5,6-epoxy-5,6-dihydro-1,10-phenanthroline platinum(II) complex exhibits promising antitumor effects *in vitro* when tested on mouse breast cancer cells (4T1), mouse colorectal carcinoma cells (CT26), human colorectal carcinoma cells (HCT116). The tested complex induced programmed cell death (apoptosis), reduced the proliferation rate, and slowed down the migration of tumor cells. The same complex reduced primary tumor growth *in vivo* and exhibited decreased hepatotoxicity, nephrotoxicity, and cardiotoxicity (DIMITRIJEVIĆ STOJANOVIĆ *et al.*, 2022). These observations show that platinum(II) complex displays promising antitumor effects against breast and colorectal carcinomas, making them potential candidates for cancer therapy. Since platinum-based drugs affect the host immune system the aim of this study was to analyze immunomodulatory effects of this platinum(II) complex.

MATERIALS AND METHODS

Preparation of [Pt(Me$_2$-mal)(5,6-epoxy-1,10-phen)]·2H$_2$O complexes

The platinum(II) complex [Pt(Me$_2$-mal)(5,6-epoxy-1,10-phen)]·2H$_2$O was synthesized using an already-known technique (DIMITRIJEVIĆ STOJANOVIĆ *et al.*, 2022). Me$_2$-mal is a bidentate-coordinated anion of 2,2-dimethylmalonic acids, whereas 5,6-epoxy-1,10-phen is a bidentate-coordinated 5,6-epoxy-5,6-dihydro-1,10-phenanthroline. A solution of 105.6 mg (0.25 mmol) of *cis*-[PtCl$_2$(DMSO)$_2$] and an equimolar amount of the disilver salt of 2,2-dimethylmalonic acids in 15 mL water was stirred at room temperature in the dark overnight. After filtering the precipitate (AgCl), the pale yellow solution of [Pt(Me$_2$-mal)(DMSO)$_2$] was utilized to prepare [Pt(Me$_2$-mal)(5,6-epoxy-1,10-phen)]·2H$_2$O. A solution of [Pt(Me$_2$-mal)(DMSO)$_2$] was treated with an equimolar amount of 5,6-epoxy-1,10-phen (49.1 mg, 0.25 mmol). The reaction mixture was stirred and heated at 40°C for 3 hours. The amorphous powder of platinum(II) complex (Pt(II) complex) was prepared from aqueous solutions by cooling in a refrigerator, and crystals of anhydrous complex were obtained by recrystallization using a small amount of chloroform. The purity of the [Pt(Me$_2$-mal)(5,6-epoxy-1,10-phen)]·2H$_2$O complex was evaluated by elemental microanalysis, ESI-HRMS, NMR (^{1}H and ^{13}C), IR, and UV-Vis spectroscopy. The crystal structure of anhydrous [Pt(Me$_2$-mal)(5,6-epoxy-1,10-phen)] was determined using single-crystal X-ray diffraction.

Isolation of peritoneal macrophages and splenocytes

Effects of platinum(II) complex on cytokine production by peritoneal macrophages and splenocytes derived from BALB/c mice were determined. The peritoneal cavity was

washed with 5 mL of cold, sterile phosphate-buffered saline (PBS). Cells were then placed on glass Petri dishes. The dishes were disposed of nonadherent cells after 2 hours of incubation at 37°C and adherent cells were retrieved by vigorous washing with an ice-cold medium, as previously described (SCORCIAPINO *et al*., 2013). The splenocytes were isolated from BALB/c mice. The spleens were gently crushed in 5 ml of Complete medium (Dulbecco's Modified Eagle's Medium-DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L L-glutamine, 1 mmol/L penicillin–streptomycin and 1 mmol/L mixed nonessential amino acids (Sigma) and separated into single cells using cell strainer. The collected cells were then centrifuged for 5 minutes at 1500 rpm. Afterward, lysis buffer was added to remove the remaining erythrocytes followed by 5 minutes of incubation on ice. After washing twice with medium, cells were re-suspended in complete medium. The total number of cells was determined in a 1:10 dilution using a triptan blue exclusion assay. Cells with viability higher than 95% were used in all further experiments.

Cells were placed in 96 well microplates (2×105 cell/well) and divided into groups: control group with medium only, cells stimulated with LPS from *E. coli* 055:B5 (0.5 μg/ml), platinum complex treated cells, and LPS and platinum(II) complex co-treated group. Also, isolated splenocytes were divided into groups: untreated control group, ConA (0.5 μg/ml) stimulated group, platinum complex treated cells, and ConA and platinum complex co-treated cells. In all *in vitro* experiments concentration of platinum(II) complex was determined according to the IC50 value obtained from our previous study (DIMITRIJEVIĆ STOJANOVIĆ *et al.,* 2022). IC50 value was related to tumor cells, while the platinum(II) complex did not show significant cytotoxicity against non-tumor cells, such as mesenchymal stem cells (DIMITRIJEVIĆ STOJANOVIĆ *et al.,* 2022). Cells were cultured for 24 hours, at 37 °C in a humidified atmosphere of 5% CO2 and 95% air in a complete medium. After the 24-hour incubation, viability assessments using triptan blue assay confirmed that viability surpassed 90% in each well, with no difference between groups (data not shown).

Measurement of Cytokine Concentrations

Following incubation, cells were centrifuged, and the resulting supernatants were collected and kept at -80°C until the time of the assay. The measurement of cytokine concentrations was performed by using ELISA assay kits from R&D Systems (Minneapolis, MN, USA) as specified by the manufacturer. The concentration of tumor necrosis factor-α (TNF-α), interleukins (IL) IL-1β, IL-6, IL-10, and IL-12 produced by macrophages and concentration of Interferon-γ (IFN-γ), IL-1, IL-10, IL-17 and TNF-α produced by splenocytes were determined and the data are presented as mean ± standard error of the mean (SEM).

Flow cytometric analysis of peritoneal macrophages and splenocytes

Immunophenotyping was performed using flow cytometry. After pretreatment with medium only/LPS/ConA/platinum complex cells were incubated with fluorochrome-labeled anti-mouse antibodies specific for F4/80 (REA126), CD3 (cluster of differentiation 3) (OTI3E10), CD206 (857615), CD69 (H1.2F3). For intracellular staining, cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/ml, Sigma-Aldrich), ionomycin (500 ng/ml, Sigma-Aldrich), and GolgyStop (BD Pharmingen, NJ) for 4 hours and stained with fluorochrome-labeled anti-mouse antibodies specific for TNFα (REA636), IL-1β (166931), IL-6 (MQ2-13A5), IL-10 (JES5-16E3), IL-12 (12-27537), IFN-γ (XMG1.2) and IL-17 (TC11-18H10). 20.000 to 50.000 cells were acquired for FACS analysis. Flow cytometry was conducted on FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA) and the data were analyzed using FlowJo (Tree Star).

Statistical analysis

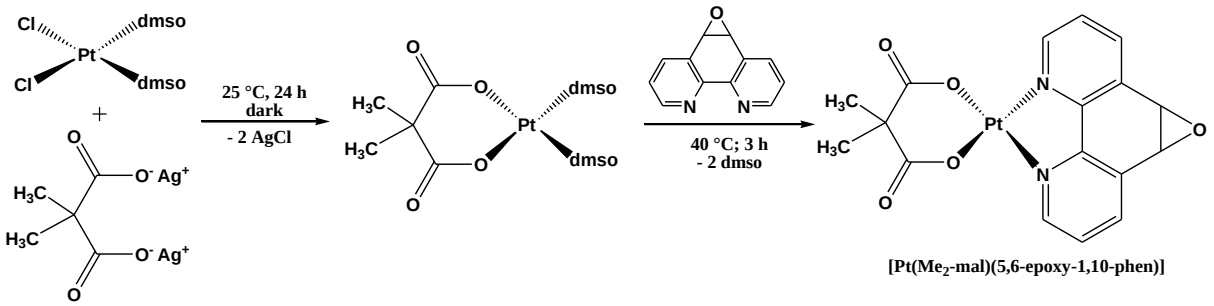
The data were analyzed using commercially available software (SPSS version 23.0). All results were analyzed using Student's t-test, Mann-Whitney U test, or Kruskal-Wallis test where appropriate. Data are presented as mean ± standard error of the mean. Statistical significance was set at $p<0.05$.

RESULTS AND DISCUSSION METHODS

Platinum-based drugs represent extensively used chemotherapeutics (RUBINO *et al.*, 2016). Drugs such as cisplatin, oxaliplatin, and carboplatin are acknowledged for their cytotoxic effects on cancer cells (ABU-SURRAH and KETTUNEN, 2006; FRONIK *et al.*, 2021). Besides their direct effect, it is also proven that platinum agents remodel the tumor microenvironment and enhance immune-mediated tumor destruction (KIM *et al.*, 2022). This is a consequence of immune checkpoint blockade, regulation of immune-system pathways, and initiation of immunogenic cell death (XUE *et al.*, 2021; KIM *et al.*, 2022; JIN *et al.*, 2024). The interaction between therapeutic and immunomodulatory effects enhances the anti-tumor efficiency of platinum chemotherapeutics, contributing to overcoming drug resistance and reducing systemic toxicity (JIN *et al.*, 2024). Our recent study has revealed that novel mononuclear 5,6-epoxy-5,6 dihydro-1,10 phenanthroline platinum(II) complex exhibits promising antitumor effects *in vitro*, as well as *in vivo* (DIMITRIJEVIĆ STOJANOVIĆ *et al.*, 2022). The immunomodulatory role of this complex was not tested.

Preparation and characterization of [Pt(Me$_2$-mal)(5,6-epoxy-1,10-phen)]·2H$_2$O complexes

According to Scheme 1, a mononuclear platinum(II) complex [Pt(Me$_2$-mal)(5,6-epoxy-1,10-phen)] was synthesized. The platinum(II) complex has been studied using elemental analysis and several spectroscopic techniques. The spectroscopic results are consistent with those previously published for the same complex (DIMITRIJEVIĆ STOJANOVIĆ *et al.*, 2022). Figure 1 illustrates the crystal structure of the anhydrous [Pt(Me$_2$-mal)(5,6-epoxy-1,10-phen)] complex as determined by single-crystal X-ray diffraction investigation. Mononuclear platinum(II) complexes have square-planar geometry, with bidentate coordinated 5,6-epoxy-5,6-dihydro-1,10-phenanthroline and anion of 2,2-dimethylmalonic acids through two nitrogen and two oxygen atoms, respectively.



Scheme 1. Illustration of the reaction for the formation of [Pt(Me$_2$-mal)(5,6-epoxy-1,10-phen)] complex.

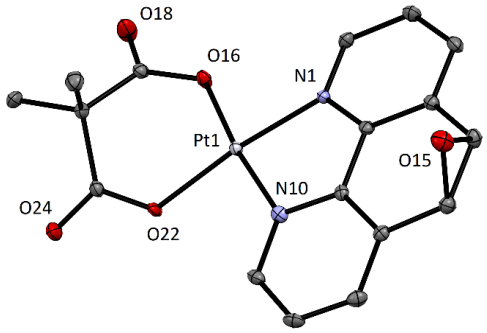


Figure 1. Crystal structure of anhydrous [Pt(Me_2-mal)(5,6-epoxy-1,10-phen)] complex (DIMITRIJEVIĆ STOJANOVIĆ *et al.*, 2022)

Our first goal was to evaluate cytokine release from peritoneal macrophages/splenocytes after LPS/ConA/platinum(II) complex incubation. LPS is a known macrophage activator. It is a molecule found in the outer membrane of gram-negative bacteria that can trigger a strong inflammatory response when detected by the innate immune cell receptors, specifically Toll-like receptors type 4 (TLR4) (AARON *et al.*, 2012; TI *et al.*, 2015). Cytokines represent small proteins involved in cell signaling and immune responses (JØRGENSEN *et al.*, 2002). We tested key proinflammatory IL-1β, TNF-α, IFN-γ, IL-6, IL-12, IL-17, and anti-inflammatory IL-10 cytokines produced by various innate immunity cells. As expected, treatment with LPS only significantly increased the concentration of proinflammatory cytokines IL-1β ($p<0.01$), IL-6 ($p<0.01$), and anti-inflammatory IL-10 ($p<0.01$) in cell supernatants in comparison to untreated cells (Fig. 2). Also, LPS-stimulation increased TNF-α and IL-12 concentration, but the effect was not statistically significant (Fig. 2).

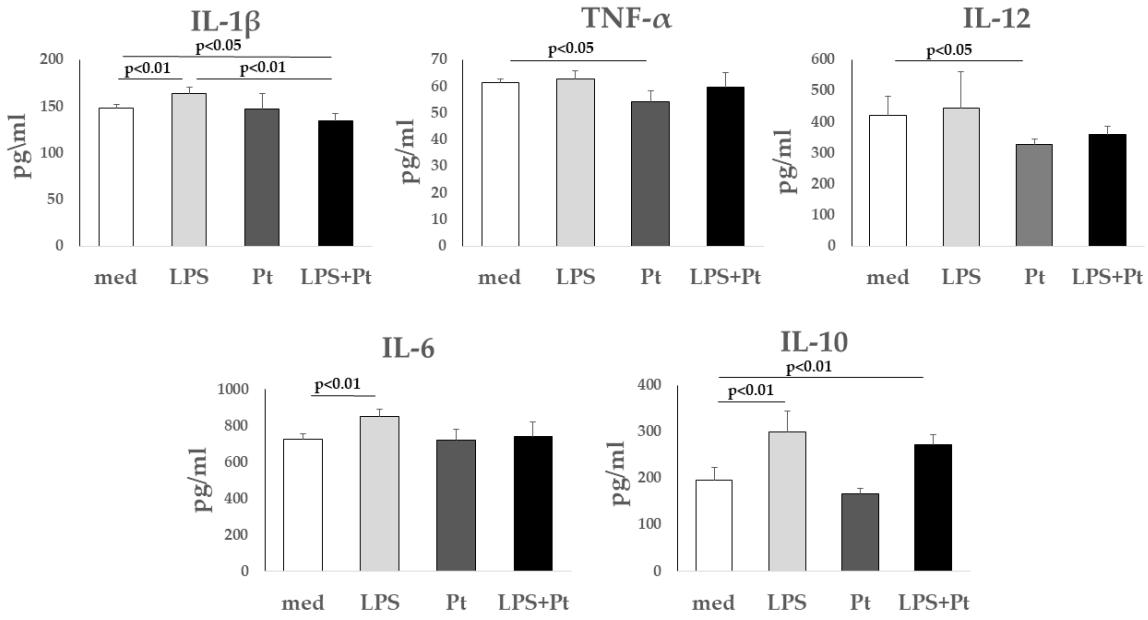


Figure 2. The concentration of cytokines produced by lipopolysaccharide/platinum(II) complex treated peritoneal macrophages. the concentration of Tumor necrosis factor α (TNF-α), interleukin (IL) 1β, IL-6, IL-10, and IL-12 were determined by ELISA. Data are shown as mean ± SEM of four mice per group and are representative of three separate experiments. Statistical significance was tested by the Mann-Whitney U test or Student's t-test, where appropriate. Med stands for medium only.

Cultivation with platinum(II) complex significantly decreased TNF-α ($p<0.05$) and IL-12 ($p<0.05$) concentration compared to untreated macrophages, as well as IL-1β, IL-6, IL-10 but the decrement of concentration of those cytokines did not reach statistical significance (Fig. 2). Finally, the cotreatment with LPS and platinum(II) complex significantly reduced IL-1β ($p<0.05$) in comparison to LPS-stimulated group. The cotreatment also decreased IL-12 and TNF-α concentration but the difference was not significant (Fig. 2). Furthermore, concentration of IL-10 was significantly increased by the cotreatment with LPS and platinum (II) complex in comparison to untreated cells ($p<0.01$) (Fig. 2). These results suggest that platinum(II) complex may modulate the macrophage's inflammatory response induced by LPS, potentially by inhibiting IL-1β and enhancing IL-10 production.

IL-1β is a proinflammatory cytokine produced by various immune cells such as macrophages and dendritic cells that promote inflammation, fever, and activation of other immune cells (LACRUZ-GUZMÁN *et al.*, 2013). IL-10 represents an anti-inflammatory cytokine that is responsible for suppressing the immune response. It is produced by various immune cells like macrophages and regulatory T- cells, and serves to prevent excessive inflammation and immune-mediated tissue damage (ATES *et al.*, 2008).

In order to fully explain the collected evidence, the functional phenotype of macrophages was analyzed. F4/80 molecule is established as a significant surface marker for murine macrophages (DOS ANJOS CASSADO, 2017). $F4/80^+$ macrophages treated with LPS increased expression of IL-1β ($p<0.05$), IL-6 ($p<0.05$), IL-10 ($p<0.05$), IL-12 ($p<0.05$), TNF-α ($p<0.05$) in comparison to untreated cells (Fig. 3). This is in line with increased concentration of IL-1β, IL-6, IL-10 in supernatant of the same cells (Fig. 2). Cultivation with platinum(II) complex significantly enhanced IL-1β ($p<0.05$), IL-6 ($p<0.05$) and TNF-α ($p<0.05$) expression compared to control group (Fig. 3). Furthermore, cultivation with platinum(II) complex did not statistically increase IL-12 and IL-10 production, and CD206 expression. The CD206 molecule is a mannose receptor primarily found on the surface of macrophages. It is used for the characterization of M2 macrophages, which have a role in reparation processes and anti-inflammatory cytokine production (ABUMAREE *et al.*, 2013). Cocultivation with LPS and platinum(II) complex significantly decreased expression of IL-1β ($p<0.05$), IL-6 ($p<0.05$), IL-12 ($p<0.05$) and TNF-α ($p<0.05$) compared to LPS stimulated cells (Fig. 3). This is in line with reduced concentration of IL-1β in supernatant of the same cells (Fig. 2). The production of IL-10 was also reduced by cotreatment with LPS and platinum(II) complex, but the effect did not reach statistical significance. These results indicate that platinum(II) complex may intensify proinflammatory responses in LPS-stimulated macrophages and also did not affect polarization toward M2 macrophages.

Further in the research immunomodulatory effects of platinum(II) complex and ConA stimulation of splenocytes were analyzed. Treatment with ConA only, a polyclonal T-cell activator (WANG *et al.*, 2004), significantly enhanced IL-17 ($p<0.05$); TNF-α ($p<0.05$) concentration in cell supernatants in comparison to untreated cells. The increase was also detected in IL-10 concentration, but the effect was not significant (Fig. 4).

Platinum(II) complex treatment significantly decreased IFN-γ concentration ($p<0.01$) as well as IL-1β but the difference was not significant (Fig. 4). However, cotreatment with ConA and platinum(II) complex significantly increased IL-17 concentration ($p<0.05$) compared to ConA-stimulated group. Cotreatment increased IL-10 and TNF-α concentration and reduced IL-1β and IFN-γ concentration but those effects did not reach statistical significance (Fig. 4).

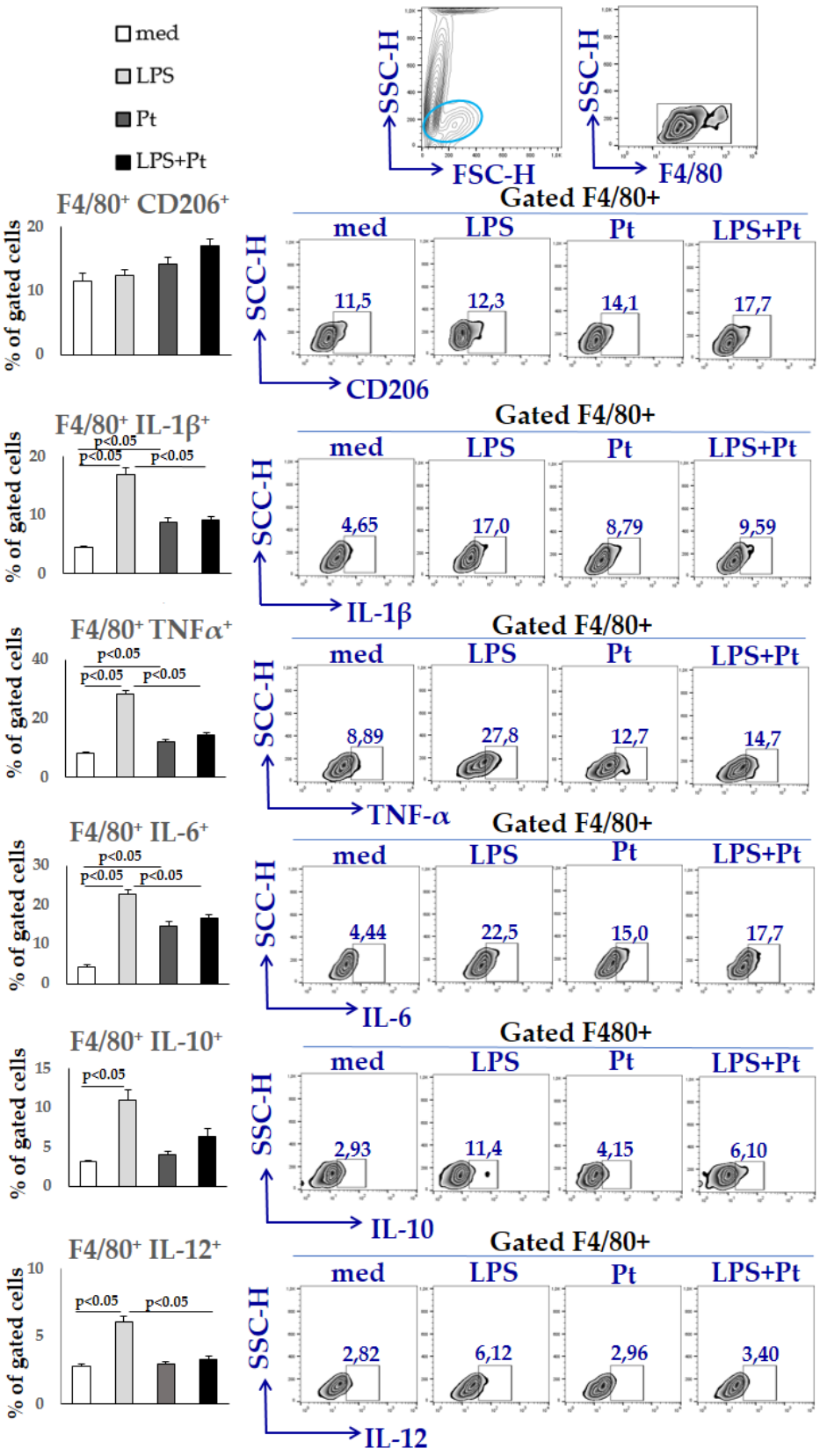


Figure 3. Expression of cytokines and markers associated with the immune response in F4/80$^+$ cells. The graphs and representative FACS plots display the percentage of F4/80$^+$ IL-1β$^+$, IL-6$^+$, IL-10$^+$, IL-12$^+$, TNF-α$^+$, and F4/80$^+$CD206$^+$ peritoneal mononuclear cells derived from BALB/C mice, attained by flow cytometry. Data are shown as mean ± SEM of four mice per group and are representative of three separate experiments. Statistical significance was tested by the Mann-Whitney U test or Student's t-test, where acquired.

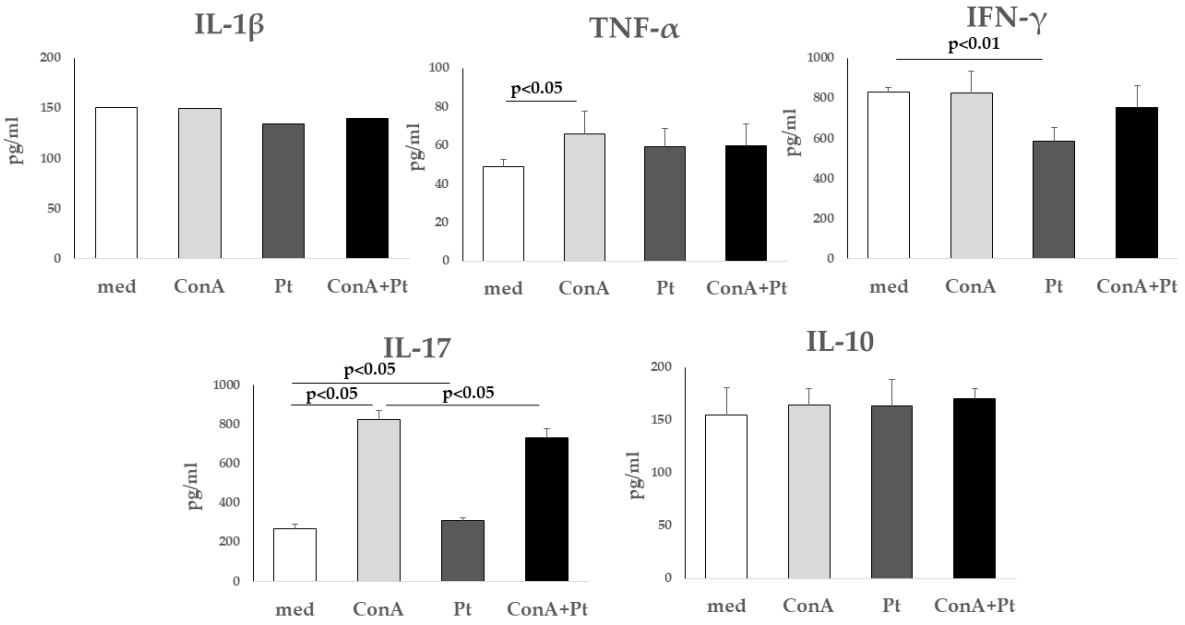


Figure 4. The concentration of cytokines produced by concanavalin A/platinum (II) complex treated splenocytes. Concentration of Interferon-γ (IFN-γ), TNF-α, IL-1β, IL-17 and IL-10 were determined by ELISA. Data are shown as mean ± SEM of four mice per group and are representative of three separate experiments. Statistical significance was tested by the Mann-Whitney U test or Student's t-test, where appropriate. Med stands for medium only.

The CD3 molecule is an integral part of the T-cell receptor complex located on T lymphocytes (ABDOEL *et al.*, 2012; KOUTNÍK *et al.*, 2022). Cultivation with ConA significantly increased the percentage of IL-1β$^+$ ($p<0.05$), IL-10$^+$ ($p<0.05$), IL-17$^+$ ($p<0.05$), IFN-γ$^+$ ($p<0.05$), TNF-α$^+$ ($p<0.05$) CD3$^+$ splenocytes, and also increased the number of CD69$^+$ CD3$^+$ splenocytes ($p<0.05$) in comparison to the control group (Fig. 5). CD69 is a transmembrane C-type lectin protein, and it is one of the earliest cell surface markers to be expressed in response to T-cell activation (ABERNETHY *et al.*, 2000). Treatment with platinum (II) complex increased percentage of IL-1β$^+$ ($p<0.05$), IL-10$^+$ ($p<0.05$), TNF-α$^+$ ($p<0.05$) CD3$^+$ splenocytes compared to untreated cells (Fig. 5). Finally, cotreatment with ConA and platinum(II) complex significantly increased number of IFN-γ$^+$ ($p<0.05$), IL-10$^+$ ($p<0.05$) CD3$^+$ splenocytes in comparison to ConA only treated cells (Fig. 5). Also, cotreatment significantly reduced number of CD69$^+$ CD3$^+$ cells compared to ConA stimulated group ($p<0.05$) (Fig. 5).

CONCLUSION

Our study has shown that LPS stimulation increased IL-1β, IL-6, IL-10, and IL-17 concentration significantly. However, co-treatment with LPS and platinum(II) complex decreased proinflammatory cytokine production compared to LPS-stimulated cells. Increment of cytokine concentration is accompanied by an increased percentage of cells exhibiting the appropriate phenotype, as is the case with IL-1β. This suggests that the platinum(II) complex has an anti-inflammatory role and that it potentially would reduce inflammation in a living organism. This indicates that mononuclear 5,6-epoxy-5,6-dihydro-1,10-phenanthroline platinum(II) complex could be used for the treatment of, not only malignancies but also inflammatory diseases in which those cytokines play a significant role in pathogenesis. For example, inflammatory bowel disease (Crohn's disease or ulcerative colitis), rheumatoid arthritis, hepatitis, multiple sclerosis, sepsis etc.

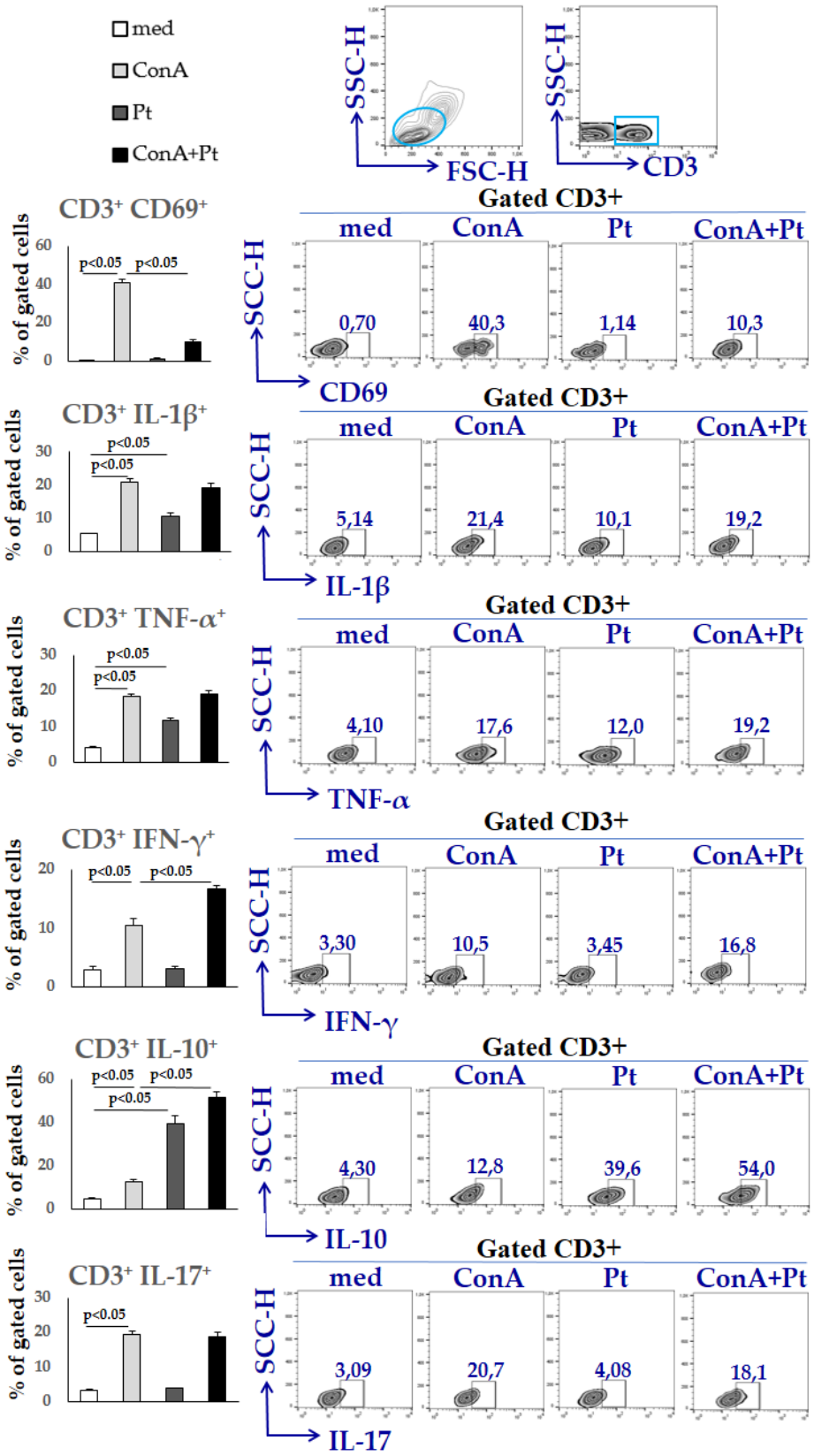


Figure 5. Expression of cytokines and markers associated with the immune response in $CD3^+$ splenocytes. The graphs and representative FACS plots display the percentage of $CD3^+IL\text{-}1\beta^+$, $IL\text{-}10^+$, $IL\text{-}17^+$, $TNF\text{-}\alpha^+$, $IFN\text{-}\gamma^+$, and $CD3^+CD69^+$ splenocytes of BALB/C mice, attained by flow cytometry. Data are shown as mean ± SEM of four mice per group and are representative of three separate experiments. Statistical significance was tested by the Mann-Whitney U test or Student's t-test, where acquired.

This platinum(II) complex could perhaps be used to modulate inflammation and reduce symptoms of these conditions. Further investigations are certainly needed to clarify the precise mechanism of action of this platinum(II) complex, but the current study serves as a foundation for a deeper understanding of its functions.

Acknowledgments

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