Article

Potential Cytotoxicity of Lipopolysaccharide Chemotypes in Ovarian Cancer Treatment

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Abstract: Ovarian cancer is the deadliest gynecological malignancy, contributing to poor overall survival rates. Our previous study suggested that lipopolysaccharide (LPS) might reduce peritoneal dissemination of ovarian cancer by enhancing cytotoxic-associated innate immunogenicity. In this study, we explored the potential cytotoxic effects of different LPS chemotypes as a novel therapeutic approach for ovarian cancer. LPS-induced chemokines are toll-like receptor 4 (TLR4)-dependent and high TLR4 levels are correlated with improved overall survival outcomes. LPS did not directly impact ovarian cancer cell proliferation. Notably, LPS chemotypes, particularly lipid A, similarly modulated chemokine expression and induced cytotoxicity of peripheral blood mononuclear cells (PBMCs) to ovarian cancer cells, highlighting the critical role of lipid A component in LPS structure. Monophosphoryl lipid A (MPLA), a detoxified derivative of LPS commonly used as a vaccine adjuvant, exhibited similar cytotoxicity of PBMC when compared to LPS and induced CCL5 and CXCL8 in PBMCs. Treatment with MPLA significantly increased survival rates in the peritoneal dissemination model of ovarian cancer, reducing tumor burden and ascites. In conclusion, detoxified lipid A derivatives, such as MPLA, appear as a promising LPS-based immunotherapeutic candidate to enhance immune cell-mediated cytotoxicity for the treatment of ovarian cancer.

Keywords: lipopolysaccharides; chemokines; peripheral blood mononuclear cells; ovarian cancer

1. Introduction

Ovarian cancer is the deadliest gynecological malignancy, with different 5-year relative survival rates varying by disease stage in the United States as follows: 92% in localized stage, 72% in regional stage, and 31% in distant stage, resulting in 51% overall survival from 2014 to 2020 [1]. Unlike most cancers that primarily spread via the bloodstream, ovarian cancer metastasizes predominantly through peritoneal dissemination, contributing to its poor overall prognosis and survival outcomes [2]. High-grade serous ovarian cancer (HGSC) is particularly aggressive, often diagnosed at advanced stages, and is associated with a worse prognosis compared to other histological subtypes [3]. A key molecular characteristic of almost all HGSC cases is the mutation of the tumor suppressor protein TP53 (TP53) [4]. Previous studies from our group have demonstrated that TP53 mutations in ovarian cancer promote the secretion of proinflammatory chemokines, which are likely to contribute to the creation of an inflammatory tumor microenvironment within the peritoneal cavity [5,6]. Based on these findings, targeting the peritoneal dissemination of ovarian cancer appears as a promising novel therapeutic strategy to inhibit metastatic ovarian cancer.

Endotoxins are ubiquitously present in the environment and are an essential component of the outer wall of Gram-negative bacteria. The active component of endotoxins, lipopolysaccharide (LPS), acts as an agonist for toll-like receptor 4 (TLR4) and can trigger both acute and chronic effects, including fever, chills, septic shock, and respiratory symptoms [7]. Our previous study has shown that LPS induces the release of proinflammatory chemokines in TLR4-positive ovarian cancer cells through TLR4-dependent activation of nuclear factor-kappaB (NF-κB) [8]. These findings were hypothesized that LPS-induced proinflammatory chemokines in ovarian cancer cells might exacerbate the peritoneal dissemination of ovarian cancer. However, our study demonstrated that LPS

actually attenuates the murine peritoneal dissemination of human SKOV-3 ovarian cancer cells [9]. Interestingly, despite these adverse effects of LPS, epidemiological evidence reveals that chronic exposures of endotoxins may reduce the risk of ovarian cancer, particularly among farm and textile workers [10–12]. Although cotton dust is contaminated with endotoxin, accurately classifying workers' exposures remains challenging due to various influencing factors, which complicates the estimation of associated health risks. Studies involving cotton textile workers (8 cohort, 1 case–cohort, and 2 case–control studies) and agricultural workers (15 cohort and 2 case–control studies) showed a negative association between endotoxin exposure and cancer risk [9]. These findings suggest a potential therapeutic role for LPS in the treatment of certain cancers, including ovarian cancer.

Despite its potent immunostimulatory properties, the toxic effects of LPS severely limit its clinical application, highlighting a critical need for safer alternatives. Monophosphoryl lipid A (MPLA), a detoxified derivative of LPS, offers a promising solution and is already marketed in Europe for allergy treatment and approved by the U.S. Food and Drug Administration (FDA) as a vaccine adjuvant [13]. Moreover, MPLA has been associated with a 25% reduction in the risk of all cancers excluding skin cancer, further underscoring its potential as a chemopreventive agent [14]. However, the structural diversity of MPLA variants, which can confer a spectrum of TLR4 agonist and antagonist activities [15], introduces uncertainty regarding their safety and efficacy profiles. Thus, a major knowledge gap remains in identifying and characterizing safe, detoxified MPLA variants that retain beneficial immunomodulatory properties without the high toxicity associated with LPS. Addressing this gap is essential to fully realize the clinical potential of LPS derivatives.

In this study, we focused on the effects of LPS chemotypes on the modulation of proinflammatory chemokine production and the cytotoxicity of peripheral blood mononuclear cells (PBMCs) against ovarian cancer cells. Specifically, we used various LPS chemotypes to identify the core structural components of LPS responsible for regulating proinflammatory chemokine expression in ovarian cancer cells. By comparing these chemotypes to their parent LPS, we aimed to determine which structural variants most effectively augmented the cytotoxic activity of PBMCs against cancer cells by comparing them with LPS-induced cytotoxicity. Furthermore, we investigated whether MPLA, a detoxified LPS derivative, could inhibit peritoneal dissemination of ovarian cancer in a murine xenograft model using human OVCAR-3 cells, which represent high-grade serous ovarian carcinoma characterized by TP53 mutation.

2. Materials and Methods

2.1. Ovarian Cancer Cell Lines and Culture

All cell lines were routinely tested for mycoplasma contamination (Lonza, Allendale, NJ, USA) following the manufacturer's protocol, with negative results confirmed. The human OVCAR-3 ovarian cancer cell line (TLR4 negative) was purchased from the American Type Culture Collection (Manassas, VA, USA). The A2780 cell line (TLR4 negative) was generously provided by Dr. Andrew Godwin (University of Kansas Cancer Center, Kansas City, KS, USA). Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with penicillin/streptomycin (100 U/mL) and 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator with 95% air and 5% CO_2. The mouse ovarian surface epithelial cancer cell line, ID8 (TLR4 positive), was kindly provided by Drs. Katherine Roby and Paul Terranova (University of Kansas Medical Center, Kansas City, KS, USA). Mouse ID8 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with penicillin/streptomycin (100 U/mL each) and 4% FBS, under the same conditions of 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. All liquid culture media were purchased from Invitrogen (Grand Island, NY, USA).

2.2. Transient Transfection and Luciferase Assay

The mouse CXCL1 promoter and its NF-B mutant constructs were generated as previously described [16]. Ovarian cancer cells were seeded in 24-well plates to approximately 75% confluence, washed, and then transiently transfected with the mouse CXCL1 promoter subcloned into the pGL3-basic luciferase reporter vector. Transfections were carried out for 16 h at 37 °C using Lipofectamine™ 2000 (Invitrogen Life Technologies, Grand Island, NY, USA). Following transfection, cells were treated with various LPS chemotypes and incubated for 16 h. After incubation, cells were washed with ice-cold PBS and lysed using lysis buffer (Promega, Madison, WI, USA). The resulting cell lysates were subsequently analyzed for luciferase activity using a microplate luminometer. Luciferase activity, expressed in relative light units, was normalized to the protein concentration in the samples.

2.3. Cell Viability Assay

Cell viability assay was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay, as previously described [9]. Following a 48-hour incubation of cells in a 24-well plate, each well was washed twice with phosphate-buffered saline (PBS). MTT solution (1 mg/mL of phenol red-free media:PBS = 4:1) was then added to each well, and the plates were incubated for 3 h in protection from light. After incubation, the MTT solution was removed, and 500 µl of isopropanol was added to each well. The plates were shaken for 10 min at room temperature to fully dissolve the formazan product. The optical density was measured at 595 nm using a microplate reader (Bio-Rad, Hercules, CA, USA), and the results were normalized to the untreated control group.

2.4. Enzyme-Linked Immunosorbent Assay (ELISA)

Tumor necrosis factor alpha (TNF) levels in the conditioned media were quantified using the Mouse TNF alpha ELISA Development Kit (#900-K54, PeproTech®, Thermo Fisher Scientific Inc., Waltham, MA USA), following the manufacturer's instructions. The optical density of each well was measured using a microplate reader set to 405 nm, with wavelength correction at 570 nm.

2.5. Overall Survival Rate of Patients with Ovarian Cancer for Toll-Like Receptor 4 (TLR4)

The Kaplan-Meier plotter database (https://kmplot.com/analysis/, accessed on 5 October 2021) was used to assess overall survival. Proportional hazards regression was applied to estimate hazard ratios (HRs) and 95% confidence intervals (CIs) based on the gene expression profile of TLR4 in 655 ovarian cancer patients from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database and The Cancer Genome Atlas (TCGA) [17].

2.6. Cytotoxicity in the Co-Culture System with Human Ovarian Cancer Cells and PBMCs

PBMCs were isolated from leukocyte filters obtained from the Red Cross Blood Bank Facility (Nashville, TN, USA) as described [18]. Leukocytes were retrieved by back-flushing the filter with sterile phosphate-buffered saline (PBS) containing 5 mM disodium EDTA and 2.5% [*w*/*v*] sucrose to elute the cells from the filter. The eluent was carefully layered onto Lymphosep™—Lymphocyte Separation Medium (1.077 g/mL) (Fisher Scientific, Pittsburg, PA, USA), and subjected to centrifugation at 1200× *g* for 30 min. Granulocytes and erythrocytes sediment at the bottom of the tube while PBMCs, which included lymphocytes and monocytes, remain buoyant on the Lymphosep™. Mononuclear cells were collected and washed with PBS (500× *g*, 10 min). After the washing step, the cells were layered on bovine calf serum and centrifuged at 400× *g* for 5 min to remove platelets. Target A2780 ovarian cancer cells (1×10^4 cells/well) were seeded into 24-cell plates. After seeding, effector PBMCs were added at the indicated effector-to-target cell ratio (1:2). The well containing both cancer cells and PBMCs was treated with or without LPS derivatives, and incubated for 48 h at 37 °C in a humidified atmosphere containing 5% CO_2. Following incubation, the cells were washed with cold PBS, and MTT (250 µL, 1 mg/mL media) was added, followed by an additional 3 h incubation of the plates. After removing the MTT solution, 500 µL of isopropanol was added to dissolve the formazan product, as described in the cell viability assay. Cytotoxicity was calculated from triplicate samples, and the absorbance rate at 595 nm was measured using a microplate reader.

2.7. Mouse Peritoneal Dissemination Model of Ovarian Cancer

A mouse peritoneal dissemination model was conducted in accordance with the guidelines approved by the Institutional Animal Care and Use Committee at Meharry Medical College (protocol 080825DSS172) and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. CD-1 nude mice (Crl:CD1-Foxn1nu; Stock No: 003814), which are T-cell deficient, were obtained from Charles River (Wilmington, MA) for human cancer cell xenograft studies. The mice were maintained in a specific pathogen-free facility at 22 °C ± 2 °C, 40%–60% humidity, and a 12:12 light:dark cycle. Human OVCAR-3 ovarian cancer cells (3×10^6 cells/mouse in 0.2 mL PBS) were intraperitoneally injected into the mice as previously described [9]. Four days post-injection, MPLA (0.5 mg/kg of body weight) was administered intraperitoneally three times per week for three weeks under the same condition as did in the previous study [9]. Body weights were monitored, and mice were euthanized upon the irreversible accumulation of ascites (approximately 8–10 mL). Health assessments were conducted three times weekly, evaluating signs such as hunched posture, lethargy, inactivity, impaired ambulation, shallow or labored breathing, changes in coat condition and weight fluctuations. Mice exhibiting clinical signs of ascites, characterized by continuous body weight increase and altered appearance or activity, were monitored daily. When a 20% increase in body weight was observed, accompanied by extensive ascites accumulation and reduced

activity, animals were euthanized for humane reasons. The survival times between the control and MPLA-treated groups were compared. Tumor burdens were examined by measuring ascites volume and tumor weight in the omentum and the diaphragm.

2.8. Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM) and were analyzed by Student's *t*-test and one-way analysis of variance (ANOVA) to assess statistical significance ($p < 0.05$). If ANOVA indicated significant differences ($p \leq 0.05$), post hoc pairwise comparisons were performed using Tukey's test to identify specific group differences. Survival data were analyzed using the log-rank test [19].

3. Results

3.1. LPS Induces CXCL1 Upregulation and TNF Secretion without Affecting Cell Viability in Mouse ID8 Ovarian Cancer Cells

In our previous work, we demonstrated that LPS induced a robust upregulation of CXCL1 in mouse ID8 cells expressing TLR4 but has no effect on human SKOV-3 ovarian cancer cells lacking TLR4 [8]. In this study, we aimed to investigate whether LPS influences CXCL1 promoter activity through an NF-κB-dependent mechanism. The mouse CXCL1 promoter contains three NF-κB binding sites [16]. We further sought to identify which specific κB site in the CXCL1 promoter mediate the response to LPS. LPS significantly increased luciferase activities of the CXCL1 promoter, as well as the κB mutation distal sites m2 and m3, in ID8 cells (Figure 1A). In contrast, the mutation at the proximal κB site (m1) did not respond to LPS stimulation (Figure 1A). This suggests that the proximal κB site plays a crucial role in the regulation of CXCL1 promoter activity in response to LPS, while the distal two κB sites contribute to its full activation. Additionally, we observed that TNF, a proinflammatory cytokine, was secreted at elevated levels in response to LPS in ID8 cells (Figure 1B). However, neither LPS nor TNF had any significant effect on cell viability in these cells (Figure 1C). To further explore the relevance of TLR4 in ovarian cancer, we analyzed overall survival data from 655 ovarian cancer patients using the Kaplan-Meier Plotter database (https://kmplot.com/analysis/ accessed on 5 October 2021), incorporating data from the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA). Notably, TLR4 expression was associated with improved overall survival in ovarian cancer patients (Figure 1D).

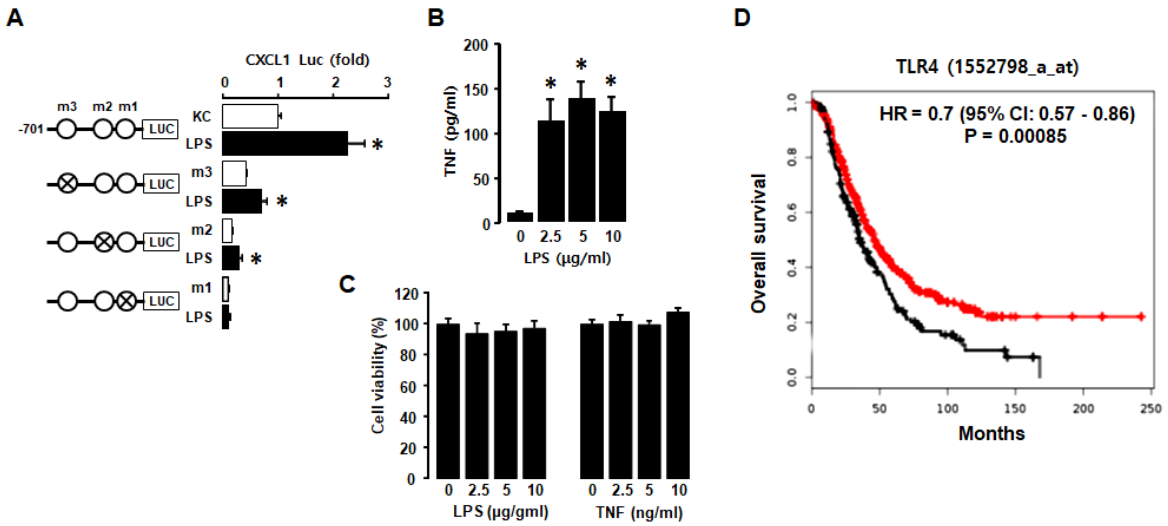

Figure 1. Effects of LPS on CXCL1 regulation, TNF production, and cell viability in mouse ID8 ovarian cancer cells. (**A**) LPS-induced CXCL1 promoter activity in mouse ID8 ovarian cancer cells. The circles represent NF-κB binding sites, while the crosses (×) indicate NF-κB mutations (m1, m2, and m3). * Denotes statistical significance ($p < 0.05$, n = 3). Luciferase activity was normalized to the protein concentration in the samples. KC promoter without NF-κB mutations was used as a basal value (1-fold) for no treatment of LPS. (**B**) LPS-induced TNF secretion measured by ELISA after 12 h of incubation with LPS in ID8 cells (n = 3). Data were expressed as mean ± SEM. (**C**) Effects of LPS and TNF on cell viability in mouse ID8 ovarian cancer cells. Cells were incubated for 48 h after LPS or TNF treatments (n = 3). (**D**) Kaplan-Meier plot showing overall survival for TLR4 in ovarian cancer patients (n = 655). Hazard ratios (HRs) were calculated using the GEO and TCGA datasets available through

the Kaplan-Meier plotter database (https://kmplot.com/analysis/ accessed on 5 October 2021). Black and red lines represent low and high levels of TLR4 expression, respectively.

3.2. Lipid A Component of LPS Chemotypes Is Critical for CXCL1 Regulation and Cytotoxicity in Ovarian Cancer Cells

The structure of LPS consists of a polysaccharide (PS) component containing the O-antigen and core oligosaccharide, as well as lipid A moiety (Figure 2A) [20]. We conducted a series of experiments to assess the differential biological activity of various LPS chemotypes, such as intact S-LPS, O-antigen-deleted Ra-LPS, O-antigen/core oligosaccharide-deleted Re-LPS, and PS-LPS, in regulating the CXCL1 promoter in ID8 cells. PS-LPS had no effect on CXCL1 promoter activity (Figure 2B), while both S-LPS and Ra-LPS induced higher CXCL1 promoter activity compared to Re-LPS (Figure 2C). These results indicate that the structural difference among LPS chemotypes influence CXCL1 regulation. We further investigated the involvement of TLR4 signaling by treating cells with CAY10614 (a lipid A antagonist) and TAK-242 (a TLR4 antagonist). CAY10614 (20 and 40 μM) and TAK-242 (100 and 250 μM) were pretreated 0.5 h prior to S-LPS treatment. Both antagonists blocked S-LPS-induced CXCL1 promoter activity in a dose-dependent manner (Figure 2D). This highlights the critical role of TLR4-mediated signaling in the regulation of CXCL1. Additionally, we assessed the immune cytotoxicity of LPS chemotypes in human A2780 ovarian cancer cells using a co-culture system with PBMC. Co-culture with PBMCs resulted in decreased cancer cell viability compared to single-cell cultures. Importantly, all LPS chemotypes, S-LPS, Ra-LPS, and Re-LPS, increased cytotoxicity in a dose-dependent manner (Figure 2E), highlighting the critical role of lipid A in mediating TLR4-driven immune responses and cytotoxicity.

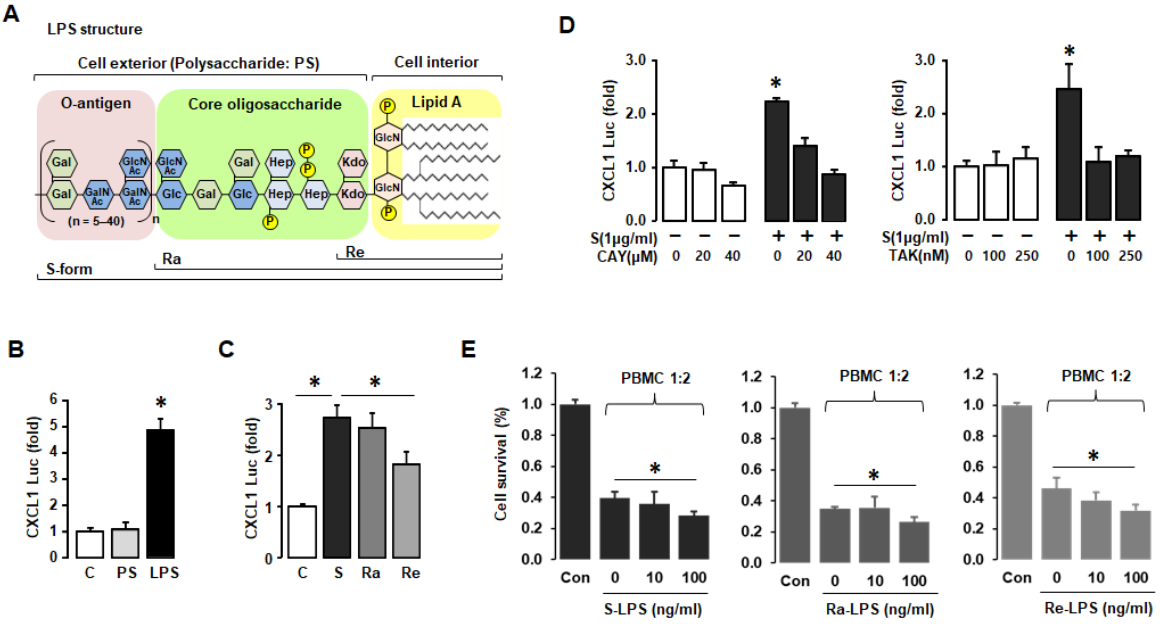

Figure 2. Differential effects of LPS chemotypes on CXCL1 regulation and cytotoxicity in ovarian cancer cells. (**A**) Structural overview of LPS chemotypes. S-form contains the O-antigen, core oligosaccharide, and lipid A moiety; Ra-form contains the core oligosaccharide and lipid A moiety; Re-form contains a part of core oligosaccharide and a lipid A moiety. (**B**) Comparison of PS and LPS on CXCL1 promoter activity in mouse ID8 ovarian cancer cells. (**C**) Differential effects of S-LPS, Ra-LPS, and Re-LPS on CXCL1 promoter activity in mouse ID8 ovarian cancer cells. (**D**) Inhibitory effects of CAY10614 (CAY) and TAK-242 (TAK) on S-LPS-induced CXCL1 promoter activity in mouse ID8 ovarian cancer cells. CAY10614 and TAK-242 were pretreated 0.5 h prior to S-LPS treatment. (**E**) Cytotoxicity of S-LPS, Ra-LPS, and Re-LPS in human A2780 ovarian cancer cells in a co-culture system with PBMC. *: $p < 0.05$ (n = 3).

3.3. Monophosphoryl Lipid A (MPLA), a Detoxified Lipid A Derivative, May Induce Cytotoxicity in Human Ovarian Cancer Cells, Probably Through Chemokine Regulation in Immune Cells

We analyzed the protein levels of chemokines, TLR-related genes, and cytokines in human immune cells using the proteomic database (http://www.immprot.org/ accessed on 3 Oct. 2019) [21]. NK and CD8 T cells exhibited elevated levels of PRF1, a marker associated with cytotoxicity, while monocytes and dendritic cells

predominantly expressed proinflammatory cytokines, including IL-1 (Figure 3A). Given the potential side effects of LPS, which present a significant barrier to its clinical use, we compared the cytotoxicity of LPS and MPLA, a low-toxicity derivative of LPS. Both LPS and MPLA induced potential cytotoxicity in a dose-dependent manner, whereas PS-LPS had no effects (Figure 3B), highlighting a critical role of lipid A. Additionally, MPLA treatment resulted in increased levels of CCL5 and CXCL8 and decreased CXCL10 levels compared to the control in PBMCs (Figure 3C). These results suggest that MPLA, a detoxified lipid A derivative, can induce immune cell-mediated cytotoxicity against ovarian cancer cells, mimicking the effects observed with LPS treatment.

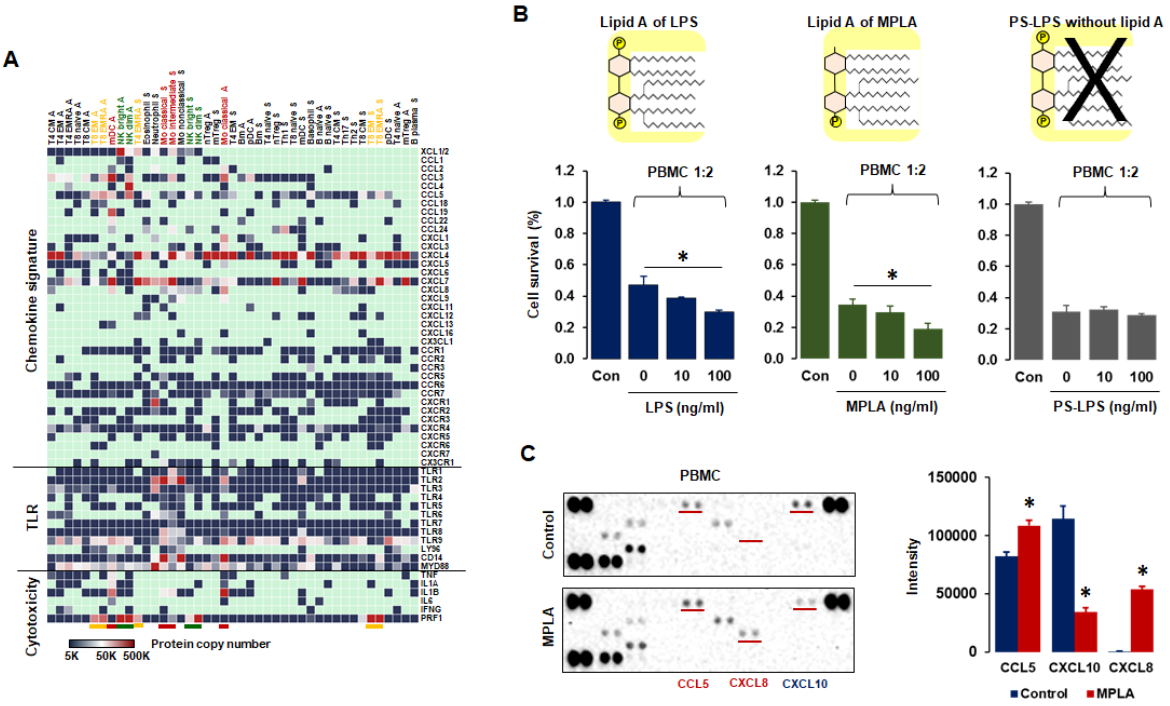

Figure 3. Proteomic analysis of chemokines and cytokines in human immune cells, cytotoxicity of LPS derivatives in human ovarian cancer cells, and MPLA-regulated chemokines in PBMCs. (**A**) Heatmap displaying the copy number of target proteins in activated and steady-state human immune cells in the context of absolute abundance. Green lines and letters: NK cells, yellow lines and letters: CD8 T cells, red lines and letters: monocytes (Mo) or dendritic cells (DC). Bright green blocks in the heatmap indicate data that were not applicable. (**B**) Cytotoxicity of LPS, MPLA, and PS-LPS in human A2780 ovarian cancer cells co-cultured with PBMC (n = 3). Both cancer cells and PBMCs were cotreated with or without LPS, MPLA, and PS-LPS. (**C**) Chemokine levels in PBMCs treated with MPLA (10 ng/mL) for 6 h, as measured by the Cytokine Proteome Profiler™ Antibody Arrays. The pictures shown are representative of duplicate experiments. *: $p < 0.05$ compared to control.

3.4. MPLA Attenuates Tumor Burden in CD-1 Mice Bearing Human OVCAR3 Ovarian Cancer Cells

Our previous study demonstrated that LPS increased overall survival and attenuated peritoneal dissemination in human SKOV-3 ovarian cancer cells [9]. However, LPS has a critical limitation for clinical use due to its endotoxin side effects. A common molecular alteration in over 95% of high-grade serous ovarian cancer cases is a mutation in TP53, although SKOV-3 cells are TP53-deficient [22,23]. In this study, we used MPLA and human OVCAR-3 ovarian cancer cells, which harbor TP53 mutation, to investigate whether MPLA could attenuate peritoneal dissemination of ovarian cancer. MPLA-treated mice exhibited significantly longer survival compared to control mice: 112 ± 4 days in control and above 140 ± 4 days in the MPLA group (Figure 4A). MPLA-treated mice also showed a reduced accumulation of ascites in the peritoneal cavity compared to the control group (Figure 4B). While there was no significant difference in tumor burdens on the omentum between the control and MPLA groups (Figure 4C), MPLA-treated mice had significantly reduced tumor weight on the diaphragm compared to controls (Figure 4D). Additionally, the peritoneal dissemination of OVCAR-3 ovarian cancer cells was reduced in MPLA-treated mice, with smaller and fewer tumors on the diaphragm compared to control mice (Figure 4E).

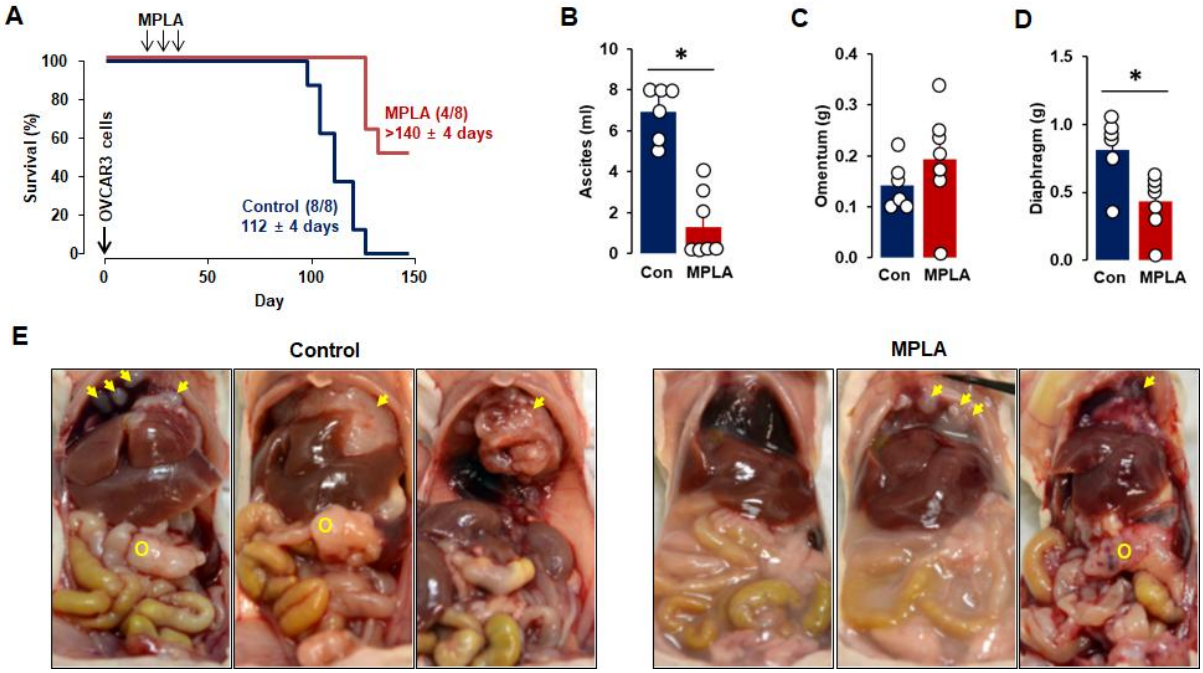

Figure 4. Beneficial effects of MPLA on tumor burden in CD-1 mice bearing human OVCAR3 ovarian cancer cells. (**A**) Survival rates of control and MPLA-treated mice (8 mice/group) by termination. Log-rank *p*-value = 0.04 with 140 end days. Comparison of tumor burden in ascites (**B**), the omentum (**C**), and the diaphragm (**D**) between control (n = 6) and MPLA-treated mice (n = 7). Ascites were withdrawn by inserting a sterile needle (20 gauge) attached to a syringe into the abdominal cavity. Tumors from the omentum and the diaphragm were weighed after dissection. Two mice in control and one mouse in MPLA groups were excluded from comparison of tumor burden because of death before euthanasia. *: $p < 0.05$. (**E**) Tumor burden imaging in the peritoneal cavity of OVCAR3 cell bearing CD-1 mice. O: omentum; yellow arrows indicate tumor burden on the diaphragm.

4. Discussion

Our primary finding is that MPLA attenuates peritoneal dissemination of ovarian cancer by reducing tumor burden and ascites, thereby enhancing survival rates as shown in Figure 4. These results suggest that MPLA, a detoxified lipid A derivative of LPS, holds potential for clinical application in cancer treatment by overcoming the side effects typically associated with LPS. Consistent with these findings, our previous study demonstrated that LPS extended survival in a mouse model of ovarian cancer [9]. Furthermore, a combination of detoxified *Salmonella* endotoxin and pyridine-extracted fraction of cell-free *Propionibacterium acnes* has been shown to improve long-term survival in a mouse ovarian cancer model [24].

The antitumor roles of LPS on cancer cells remain controversial. Some studies have shown that LPS or its synthetic lipid A derivative inhibits growth in various cancers, including colorectal cancer [25], prostate cancer [26], melanoma [26], fibrosarcoma [27], glioblastoma [28], hepatoma [27,29], lung cancer [27], and pancreatic cancer [30]. However, no effects were observed in fibrosarcoma and mammary adenocarcinoma [29]. Figure 1C showed that LPS and its induced TNF did not directly affect the viability of mouse ID8 cells, which are TLR4 positive. Notably, Figure 1D showed that high TLR4 expression correlated with better overall survival in patients with ovarian cancer. These findings suggest that the effects of LPS on ovarian cancer cells may be indirect. Additionally, LPS has been shown to enhance the adhesion of breast cancer cells to monocytes [31], while the combination of LPS and interleukin-4 (IL-4) inhibited the invasion of pancreatic cancer cells in co-culture with macrophages [32]. These observations suggest that LPS may inhibit cancer progression through its activated immune cells.

Since LPS induces keratinocyte chemoattractant (mouse CXCL1-3 chemokines) in TLR4-positive ID8 cells [8], Figure 1A confirmed that the proximal κB site in CXCL1 promoter is critical for LPS-induced regulation of CXCL1, as previously described in proinflammatory cytokine-induced CXCL1 expression [16,33]. Figure 2 shows that the comparative effects of LPS chemotypes on CXCL1 regulation and the cytotoxicity of PBMCs toward ovarian cancer cells highlight the critical role of the lipid A component in LPS structure. However, the therapeutic application of LPS chemotypes as antitumor immunotherapeutic agents remains premature in cancer research, including ovarian cancer. Therefore, a comprehensive evaluation of the ideal LPS chemotype, considering

structural, functional, and adjuvant factors, is essential for targeting peritoneal dissemination, a specific metastasis pattern in ovarian cancer.

TLR4 antagonists have been developed as potential treatments for sepsis and chronic inflammatory diseases, while TLR4 agonists are explored as vaccine adjuvants or immunotherapeutics [34]. Figure 4B showed that MPLA, a detoxified lipid A derivative of LPS, reduced the accumulation of ascites as a main characteristic of ovarian cancer as well as tumor burden on the diaphragm. These findings support the potential of MPLA as an LPS-based immunotherapeutic for ovarian cancer. LPS or its lipid A analogs have shown promise in stabilizing cancer progression in various cancer types, as demonstrated in small clinical trials [35]. Intradermal LPS treatment was less toxic and elicited antitumor effects when combined with cyclophosphamide in advanced cancer patients [36]. Oral LPS appeared to have clinical effects on malignant lung and breast cancers without significant side effects [37]. These results suggest that alternative routes to administration may reduce LPS-associated side effects in ovarian cancer. A conjugate of paclitaxel and LPS showed higher antitumor activity and improved survival compared to paclitaxel and LPS alone in melanoma-bearing mice [38]. Furthermore, the combination of LPS and cyclophosphamide inhibited pulmonary metastasis in lung cancer-bearing mice [39]. Although these findings are based on mouse models, they support the potential benefits of combining MPLA with conventional ovarian cancer therapies. Compared to LPS, MPLA demonstrated similar cytotoxicity in PBMCs, inhibiting cancer cell viability and inducing CCL5 and CXCL8 in PBMC as shown in Figure 3. Thus, MPLA may activate immune cell-mediated antitumor effects, leading to reduced tumor burden and improved overall survival. Further studies are needed to identify the specific immune cells involved in MPLA-mediated inhibition of peritoneal dissemination in ovarian cancer with immune-mediated mechanism of MPLA. Although being immunologically potent, LPS is too toxic for clinical applications to induce systemic inflammation, pyrogenicity, and endotoxic shock [40]. Therefore, MPLA may be used for a clinically viable immunostimulant with a safety and regulatory approval for human use, particularly as a vaccine adjuvant [13], offering the opportunity for development of detoxified lipid A derivatives without significant side effects [40].

In conclusion, detoxified lipid A derivatives of LPS, such as MPLA, appear as a promising agent for LPS-based immunotherapeutics in ovarian cancer by enhancing the cytotoxicity of immune cells.

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Institutional Review Board Statement: The study was conducted according to the NIH guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Meharry Medical College (080825DSS172 and date of approval: 8 October 2009).

Informed Consent Statement: Not applicable.

Data Availability Statement: Kaplan-Meier plotter database (https://kmplot.com/analysis/ accessed on 5 October 2021) is available based on gene expression profile of TLR4 in 655 ovarian cancer patients from Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database and The Cancer Genome Atlas (TCGA).

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